Phenotypic and molecular characterization of the carbapenem resistant *Klebsiella pneumoniae* and *Escherichia coli* causing blood stream infections

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be held in April 2015

Certificate

This is to certify that the dissertation entitled "Phenotypic and molecular characterization of the carbapenem resistant *Klebsiella pneumoniae* and *Escherichia coli* causing blood stream infections" is a bonafide work done by Dr. Archa Sharma towards the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R. Medical University, to be held in April 2015.

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Declaration

I hereby declare that this M.D. Dissertation titled "**Phenotypic and molecular characterization of the carbapenem resistant *Klebsiella pneumoniae* and *Escherichia coli* causing blood stream infections**" is the bonafide work done by me under the guidance of Dr.V.Balaji, Professor and HOD, Department of Clinical Microbiology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

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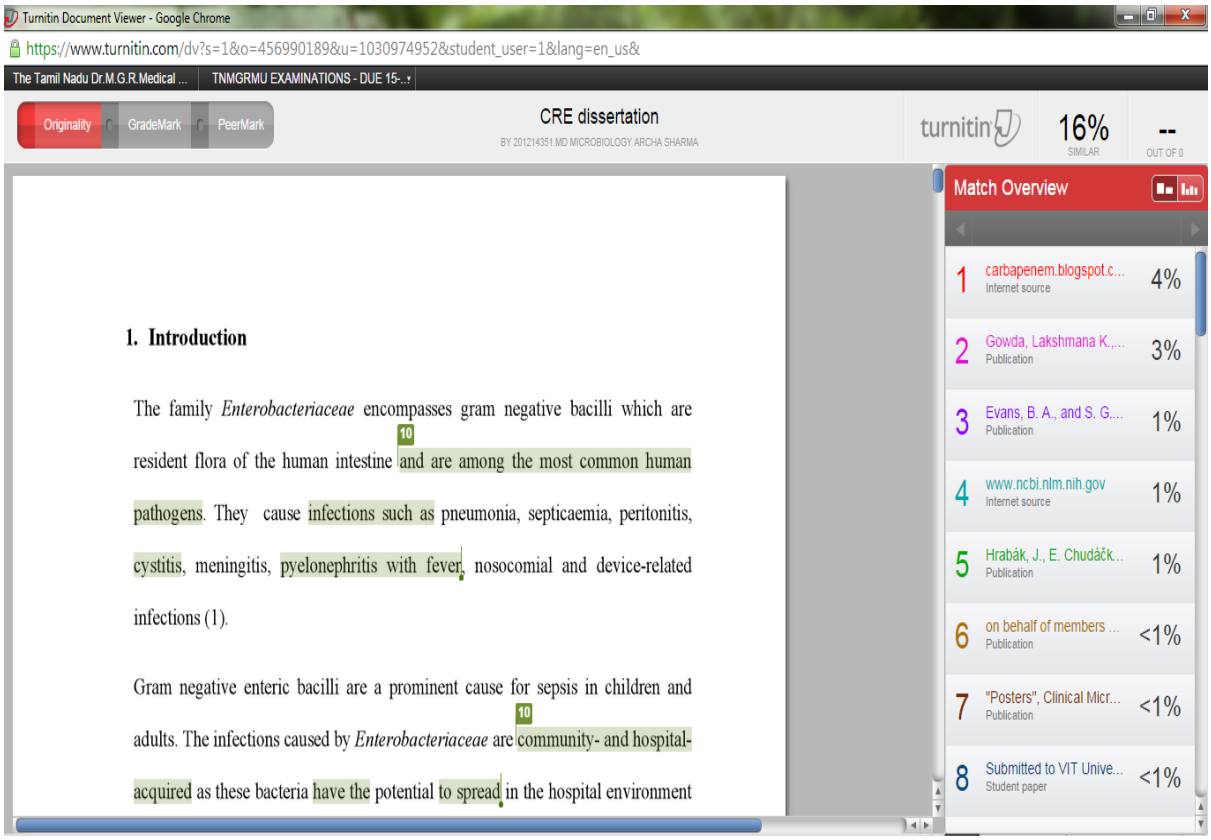
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1. Introduction
The family Enterobacteriaceae encompasses gram negative bacilli which are resident flora of the human intestine and are among the most common human pathogens. They cause infections such as pneumonia, septicaemia, peritonitis, cystitis, meningitis, pyelonephritis with fever, nosocomial and device-related infections (1).
Gram negative enteric bacilli are a prominent cause for sepsis in children and adults. The infections caused by Enterobacteriaceae are community- and hospital-acquired as these bacteria have the potential to spread in the hospital environment
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Acknowledgement

"God Almighty, without whose blessings and divine presence this dissertation would not have been possible"

I express my respect and gratitude to myesteemed and illustrious teacher and guide Prof. Dr. V.Balaji, for being a constant source of inspiration and showing immense patience with me. I am grateful for his never-ending support both academic and non-academic.

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-My husband and family for all the love, understanding and for always being there.

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February 18, 2013

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Sub: **FLUID Research grant project NEW PROPOSAL:**
Phenotypic and molecular characterization of the β-lactamase and carbapenemase producing Enterobacteriaceae causing blood stream infections. Dr. Archa Sharma, P.G Registrar, Emp. No. 20848, Clinical Microbiology, Dr. V. Balaji, Emp. No.30033, Clinical Microbiology, Dr. Thambu David, Emp. No.30008, General Medicine, Dr. Santhanam Sridhar, Emp. No.30531, Neonatology, Dr. Winsley Rose, Emp. No..28222, Paediatrics, Dr.Shalini Anandan, Emp. No. 30523, Clinical Microbiology, Mr. Ranjith, Emp. No32802, Mr. Lionel Jones, Emp. No.52995, Clinical Microbiology.

Ref: IRB Min No: 8201 dated 13.02.2013

Dear Dr. Archa Sharma,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

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Dear Dr. Archa Sharma,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Phenotypic and molecular characterization of the β-lactamase and carbapenemase producing *Enterobacteriaceae* causing blood stream infections." on February 13, 2013.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Proforma
3. Cvs of Drs. Archa Sharma, V. Balaji, Thambu David, Santhanam Sridhar, Winsley Rose, Shalini Anandan, Mr. Ranjith, Mr. Lionel Jones.
4. A CD containing documents 1 – 3

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The following Institutional Review Board (Research & Ethics Committee) members were present at the meeting held on February 13, 2013 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerlogy & Leprosy, CMC.	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Surgery (Colorectal), CMC.	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS MCh	Professor, Neurological Sciences, CMC	Internal, Clinician
Dr. P. Prasanna Samuel	B.Sc, M.Sc, PhD	Professor Dept. of Biostatistics, CMC	Internal, Statistician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Dept. of Pulmonary Medicine, CMC.	Internal, Clinician
Dr. Simon Rajaratnam	MBBS, MD, DNB (Endo), MNAMS (Endo), PhD (Endo), FRACP	Professor, Endocrinology, CMC	Internal, Clinician
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Dr. Chandrasingh	MS, MCH, DMB	Urology, CMC	Internal, Clinician
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Mr. Joseph Devaraj	BSc, BD	Chaplain, CMC	Internal, Social Scientist
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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs. 60,000/- (Rupees Sixty Thousand only) will be granted for 18 months.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. V. Balaji, Department of Clinical Microbiology

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Table of contents

Abstract

Title of the abstract:

Phenotypic and molecular characterization of the carbapenem resistant *Klebsiella pneumoniae* and *Escherichia coli* causing blood stream infections

Department: Department of Clinical microbiology

Name of the candidate: Dr.Archa Sharma

Degree and subject: M.D., Microbiology

Name of the guide: Dr.V.Balaji

Objectives:

Phenotypic and molecular characterization of the carbapenemase producing *Klebsiella pneumoniae* and *Escherichia coli* causing blood stream infections by

a. Phenotypic differentiation of carbapenemase and non-carbapenemase mediated mechanisms by the CarbaNP test.
b. Molecular characterization ofthe isolates for bla_{NDM}, $bla_{\text{KPC,}}$ $bla_{\text{OXA48 like}}$, bla_{IMP}, bla_{VIM} genesby multiplex-PCR.
c. To sequence a proportion of the positive isolates to determine the enzyme variants.

Methods:

During the study period 122 consecutive non- duplicate carbapenem resistant *Klebsiella pneumoniae* and *Escherichia coli* isolates causing blood stream infection were included in the study. The isolates were identified by cultural and biochemical characteristics. Carbapenemresistance was tested by imipenem and meropenem disk diffusion testing. CarbaNP test was done for the detection of carbapenemase production.Molecular characterization of the carbapenem resistant isolates was performed using amultiplex-PCR forbla_{NDM}, bla$_{\text{KPC,}}$ $bla_{\text{OXA48 like}}$, $bla_{\text{IMP}}$and bla_{VIM}

genes. 20 random isolates positivefor*bla*$_{\text{OXA48 like}}$ and 7 isolates positive for*bla*$_{\text{NDM}}$ genes were sequenced to identify the enzyme variants.

Results:

Of the 122 isolates 97% were positive for carbapenemase production by CarbaNP test. *bla*$_{\text{NDM}}$ was found to be the predominant gene seen in 40% of the isolates. This was followed by *bla*$_{\text{OXA48like}}$ gene in 39%. 12% of the strains showed co-existence of both *bla*$_{\text{NDM}}$ and*bla*$_{\text{OXA48like}}$genes.None of the isolates showed *bla*$_{\text{VIM,}}$ *bla*$_{\text{KPC}}$and *bla*$_{\text{IMP}}$genes.All the 20 (100%) of the *bla*$_{\text{OXA48 like}}$ genes were found to be *bla*$_{\text{OXA181}}$ a variant of the *bla*$_{\text{OXA48 like}}$ gene. The 7 *bla*$_{\text{NDM}}$ gene positives were found to be the*bla*$_{\text{NDM 1}}$ variant.

Key words: *Klebsiella pneumoniae*, *Escherichia coli*, Carbapenem, resistance, India

1. Introduction

The family *Enterobacteriaceae* encompassesgram negative bacilli which are resident flora of the human intestine and are among the most common human pathogens. They cause infections such as pneumonia,septicaemia, peritonitis, cystitis,meningitis, pyelonephritis with fever, nosocomial and device-related infections(1).

Gram negative enteric bacilli are a prominent cause for sepsis in children and adults.The infections caused by *Enterobacteriaceae* are community- and hospital-acquired as these bacteria have the potential to spread in the hospital environment and also across continents(2)(3). Irrational use of antibiotics coupled with rapid emergence of drug resistance in bacteria is a major problem faced by health care setting around the world. The production of β-lactamase is the most common mechanism of resistance to drugs especially in gram negative bacteria(4).

Carbapenems are broad spectrum antibiotics belong to the β-lactam group.These drugs were primarily introduced in the 1980s for the treatment of ESBL producing gram-negative bacilli. Like other β-lactam antibiotics they act by inhibiting the cell wall synthesis and display a time dependent bactericidal activity. Carbapenems are highly active against most of the aerobic and anaerobic bacteria except *Entercoccus spp*, MRSA(Methicillin resistant *Staphylococcus aureus*), *Stenotrophomonas maltophilia* and members of the genera; Rickettsia, Chlamydia and Mycoplasma(5).

1.1 Carbapenemase producing gram negative bacilli

Carbapenemases are a versatile group of β-lactamases that are characterised by their resistance to virtually all β-lactam antibiotics, including the cephalosporins and carbapenems. They have also been found to be resistant to other classes of drugs like fluoroquinolones, aminoglycosides and co-trimoxazole.

The carbapenemases fall into three classes according to their amino acid sequence. (Table 1.1)

Table 1.1 Classification of carbapenemase enzyme based on amino acid sequences

Class of carbapenemases	Representative enzymes
Ambler Class A	KPC, SME, NMC-A, IMI, PER, GES, SFC,
Ambler Class B (Metallo β- lactamase)	VIM, GIM, SIM, NDM, IMP, SPM
Ambler Class D (Oxacillinases)	OXA, PSE

Abbreviations in table 1.1:

KPC - *Klebsiella pneumoniae* carbapenemase

SME - *Serratia marcescens* enzyme

NMC - Non metallo-enzyme carbapenemase

IMI - Imipenem-hydrolyzing β-lactamase

PER - Pseudomonas extended resistant

GES - Guiana extended spectrum

SFC – Serratia

VIM - Verona integron-encoded MBL

GIM - German Imipenemase

SIM - Seoul imipenemase

NDM - New Delhi MBL

IMP - Active on imipenem

SPM - Sao Paulo MBL

OXA – Oxacillinase

PSE –Pseudomonas specific enzyme

The resistance to carbapenem groups of drugs is on the rise. It is especially of concern in the nosocomial pathogens which are multidrug resistant. The most common mechanism of carbapenem resistance in *Enterobacteriaceae*is the production of carbapenemase enzymes. These enzymes are carried on mobile plasmids and have a greater propensity to cause outbreaks (6). The prevalence of carbapenem resistant *Enterobacteriaceae* in India has been reported to vary from 5.3% to as high as 51% (7–9)

Carbapenemases belonging to class A of molecular classification include KPC *(K. pneumoniae* carbapenemase), GES (Guiana extended-spectrum β- lactamases, Nmc-A (non- metallo-carbapenemase-A)/ IMI (imipenem-resistant), SME *(Serratia marcescens* enzyme), SFC (serratia fonticola carbapenemase), and BIC β--lactamases.

Plasmid mediated, Ambler class B, carbapenemases which were widely found in *Pseudomonas* and other non-fermenting gram negative bacilli are now being increasingly identified in *Enterobacteriaceae*. Metallo β- lactamases (Mβ-L) are named so because their activation requires binding of metal ions such as divalent zinc ion to specific site. Various families of Mβ-Ls are identified in different parts of the world namely Imipenemase (IMP), Verona Integron encoded Mβ-L (VIM), Sao Paulo Mβ-L (SPM), German Imipenemase (GIM) and Seoul Imipenemase (SIM). Of these, members of VIM and IMP family have a worldwide distribution. Recent identification of New Delhi metallo-β-lactamase-1 (NDM-1) producers, originally in the United Kingdom, India, and Pakistan and

now worldwide, is worrisome. NDM is now the most commonly isolated enzyme from the Indian subcontinent and is rapidly spreading worldwide (10).

Carbapenemases of the oxacillinase-48 type have been identified mostly in Mediterranean and European countries and in India. This group ofβ- lactamases is capable of hydrolysing penicillins, cloxacillin, oxacillin and are poorly inhibited by clavulanic acid and EDTA. Their prevalence is on a rise and have in recent studies found to be the predominant carbapenemases in countries like France and Belgium(11,12).

Identification of the carbapenem resistance*in vitro* relies on phenotypic methods and molecular techniques.The phenotypic tests can be used for screening of clinical isolates for the presence of carbapenem resistance, but the genotypic methods such as PCR are the confirmatory tests (13).

1.2 Need for surveillance of antibiotic resistance

The emergence of drug-resistant organisms both in the hospital environment and in the community is a major concern for health care providers. Surveillance studies have provided important information about changes in the spectrum of microbial pathogens and trends in the antimicrobial resistance patterns in both nosocomial and community-acquired infections. Continued monitoring of antimicrobial resistance patterns in hospitals is essential to guide effective empirical therapy.

Adequate detection of multidrug resistant microorganisms in the routine diagnostic laboratory is essential for patient care because

(i) it is essential for the appropriate choice of antibiotic

(ii) it guides institution of suitable hospital infection control measures as these microorganisms are associated with rapid spread/outbreaks that result in increased morbidity, mortality, prolonged hospital stay and increased treatment costs.

The present study is a descriptive study done with this background in a tertiary care centre. We intended to characterize the mechanisms of carbapenem resistance in *Klebsiella pneumoniae* and *Escherichia coli*and to study the susceptibility profile of these organisms to other classes of drugs.

2. Aim of the study

To characterize the different carbapenem resistance mechanisms in *Klebsiella pneumoniae*and *Escherichia coli*isolated from blood stream infections using phenotypic and genotypic methods.

3. Objectives

i. To identify the carbapenem resistant isolates by disk diffusion for imipenem and meropenem.

ii. To compare the antimicrobial susceptibility pattern of these isolates for first line antimicrobial agents and to determine the susceptibility of these isolates to second line antimicrobials using the disk diffusion technique.

iii. To detect the various carbapenem resistance mechanisms by performing the following tests:

   a. Phenotypic differentiation of carbapenemase and non-carbapenemase mediated mechanisms by the CarbaNP test.

   b. Molecular characterization of the isolates forbla_{NDM}, $bla_{\text{KPC,}}$ $bla_{\text{OXA48 like}}$, bla_{IMP}, bla_{VIM} genesby multiplex-PCR.

   c. To sequence a proportion of the positive isolates to determine the enzyme variants.

4. Review of literature

4.1 *Enterobacteriaceae* and blood stream infections

Enterobacteriaceae are one of the most important family of bacteria that comprises of rod shaped, gram negative bacteria which are inhabitant flora of the human intestine and are among the most common human pathogens. They cause infections such as septicaemia, pneumonia, peritonitis, meningitis, cystitis and pyelonephritis with fever, and device-associated infections both in adults and children. They account for about 50% cases of blood stream infections(14).*Escherichia coli*(*E. coli*), *Klebsiella pneumoniae*(*K. pneumoniae*) and *Enterobacter spp*. are the most frequent members of the family causing a variety of community and nosocomial infections such as urinary tract infections, lower respiratory tract infections, post-surgical intra-abdominal infections and bacteraemia(15).

Blood stream infections have a varied presentation from self-limiting infections to life threatening sepsis and necessitate prompt and aggressive therapy. The aetiology of blood stream infections includes a wide spectrum of organisms and varies among different geographical regions(16).The bacterial causes of blood stream infections includes gram positive organisms such as *Staphylococcus aureus*, coagulase negative *Staphylococcus spp*, and *Enterococcus species*; gram negative bacteria such as *E. coli, K. pneumoniae* among Enterobacteriaceae; and non-fermenting gram negative bacilli *Pseudomonas spp* and *Acinetobacter baumannii*. Blood stream infections can be community acquired or hospital acquired(17). Gram negative bacilli are becoming an important cause of blood streaminfections especially in the hospital acquired infections(18). There are many reports of an etiological shift with predominance of

gram negative bacteraemia from various countries(19–22). Increasing prevalence of gram negative bacteremia has also been reported in both adult and paediatric populations from India(23–25). The knowledge of common aetiology is essential for accurate and targeted empirical therapy in these patients.

The increasing prevalence of multidrug resistance in gram negative bacilli complicates the treatment of blood stream infections caused by these pathogens. Bacteria that were earlier susceptible can becomeresistant by acquiring plasmids from other bacteria, by mutation or by selection of resistant mutants during the course of therapy.

The widespread dissemination of Extended spectrum β- lactamase and carbapenemase producing *Enterobacteriaceae* is becoming a major concern as the therapeutic options are limited and increasing treatment failure is noted(26).

4.2 Pharmacology of carbapenems

4.2.1 Carbapenems

Carbapenems are broad spectrum antibiotics belonging to the large family of β-lactam antibiotics. Carbapenem antibiotics have a broad spectrum of activity against many gram positive and gram negative bacteria. The term carbapenem is defined as "the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3 but with the substitution of carbon for sulphur at C-1". The stereochemistry of the hydroxyl-ethyl side chain of the carbapenems is a major trait of carbapenems and is vital for their activity (fig.4.1). Resembling the other β--lactam antibiotics penicillin and cephalosporins, carbapenems act by binding to the penicillin binding proteins PBPs and inhibiting cell wall synthesis in the bacteria.Among the β-lactams,

carbapenems were considered exceptional because they were relatively resistant to hydrolysis by most β-lactamases including extended-spectrum β-lactamases (ESBLs) and AmpCβ-lactamases. Thus when introduced on 1980's, carbapenems were used as antibiotics of choice in infection with drug resistant pathogens.(5)

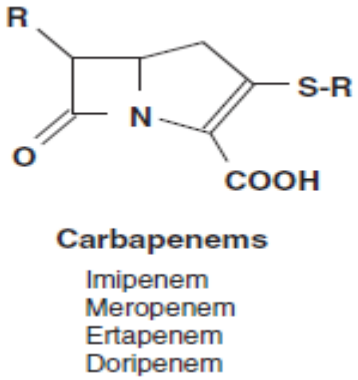


Fig.4.1. Structure of carbapenems.
Adapted from (5)

4.2.2 Mechanism of action

Carbapenems enter the bacterial cell wall through outer membrane proteins called porins as they cannot easily diffuse through the cell wall. After they enter the periplasmic space they permanently acylate the penicillin binding proteins (PBPs) such as PBP1a, 1b, 2 and 3. PBPs are enzymes that act as catalysts in the last step of trans-peptidation in synthesis of peptidoglycan resulting in the disruption of cell wall. β-lactams inhibit the crosslinking of peptidoglycan causing a build-up of precursors, that activate the autolytic enzymes for digestion of existing peptidoglycan. Consequently, the structural integrity of the cell wall decreases and bacterial lysis occurs(26).

The carbapenems available and under development are classified into 3 groups, their spectrum of activity issummarized in Table 4.1.

Table 4.1. Carbapenem antibiotics available and under development

Carbapenems	Group1	Group2	Group3
	Ertapenem	Imipenem	Tomopenem
	Panipenem	Meropenem	Razupenem
	Tebipenem	Doripenem	
		Biapenem	

4.2.3 Pharmacology

Carbapenems have similar pharmacokinetic profile but they differ in their half-lives. Imipenem is metabolized and thus inactivated by the dehydropeptidase-I (DHP-I) enzyme in the proximal tubules of the kidney. Clinically it is used in combination with a DHP-I inhibitor cilastatin in 1:1 ratio for attaining adequate concentrations in serum.

Meropenem,ertapenem and biapenem do not require concomitant administration of DHP-I inhibitor. Doripenem, imipenem and meropenem have similar pharmacokinetic properties.(27)

Meropenem and imipenem are renally excreted. Elimination of ertapenem is by acombined mechanismof conversion to a β-lactam ring-opened derivative by a hydrolytic metabolism andexcretionthrough kidney by glomerular filtration and secretion of the unchanged drug. Doripenem is excreted unchanged in the urine by glomerular filtration and tubular secretion(16).

Carbapenems are distributed primarily extracellularly and are also distributed in wide variety of tissues. The tissue concentration of these drugs is in the same range as that in the plasma(28).

Ertapenem is highly protein bound and an actual estimation of albumin is required for deciding the dosage interval of this drug. This also ensures optimal dosage in patients who are critically ill and may have hypoglobulinemia(29).

4.2.4 Spectrum of activity

Carbapenems exhibit a generally broader in vitro antimicrobial spectrum when compared to the other availableβ-lactams andβ-lactam/β-lactamase inhibitor combinations. Imipenem, doripenem and panipenem,are more potent antibiotic agents acting against Gram-positive organisms. Meropenem, ertapenem, biapenem and doripenem are slightly more effective against Gram-negative bacteria. Important aspects toconsider for carbapenem antibiotics are that ertapenem has a more limited activity when compared to imipenem or meropenem against non-fermenting gram negative bacilli such as *P. aeruginosa*. Imipenem and doripenem are more potent for *A. baumannii.* Also,doripenem is the drug in this class that is least susceptible to hydrolysisby carbapenemase enzymes(30).

Carbapenems when used in combination with other antimicrobialagents are used to treat serious infections. Combination therapy of carbapenem and other antimicrobials has become an important area of study as the MDR infections are on the rise and these pathogens requireaggressive and prompt initiation of therapy (5). The spectrum of activity of the carbapenems are summarized in table 4.2

Table 4.2 Spectrum of activity of the carbapenems

Carbapenems	**Ertapenem Panipenem Tebipenem**	**Imipenem Meropenem Doripenem Biapenem**	**Tomopenem Razupenem**
Activity against gram negative bacilli	Yes	Yes	Yes
Activity against non- fermenting gram negative bacilli	No	Yes	Yes
Activity against MRSA	No	No	Yes

4.2.5 Safety profile

β-lactam antibiotics are the most commonly prescribed class of antibiotics as they have a good safety profile when compared with other antibiotics.Anaphylaxis to β-lactam compounds in patients with allergy to these drugs can also occur with carbapenems.Other commonlyencountered adverse effects include headache, mild hypotension, localized rash and phlebitis at the injection site.Carbapenems are otherwise generally well tolerated.(27)

4.3 Mechanisms of carbapenem resistance in *Enterobacteriaceae*

In Enterobacteriaceae, resistance to carbapenems can be due to the following mechanisms:

A) Carbapenemase mediated mechanism:

(i) Acquiringgenes that encode for a β- lactamase enzymes capable of hydrolyzing carbapenems and most of the β-lactam agents.

B) Non carbapenemase mediated mechanisms:

(i) A decrease in the uptake of antibiotics bya reduction in expression of porin channels

(ii) Over-expression of efflux pumps

The porin proteins are trans-membrane ion channels which facilitate the entry of antimicrobials and other hydrophilic compounds, while efflux pumps are cell wall proteins which actively extrude the harmful substances entering into the bacterial cell(26). Contrary to carbapenemases, these mechanisms are physiological modifications and are not specific to β-lactam agents.

4.3.1 Carbapenemase mediated mechanism

In Enterobacteriaceae, the foremost mechanism involved in resistance toβ-lactams is the production of certain enzymes, termedβ-lactamases, that exhibit hydrolytic activity against β-lactam ring in the antibiotics. Depending on theiractivity for easy understanding these enzymes are grouped into four groups:

i. Penicillinases: these enzymes inactivate penicillins but are unable to degrade otherβ-lactam antibiotics such as cephalosporins, aztreonam and carbapenems.
ii. Cephalosporinases are enzymes that preferentially degrade cephalosporins and amino penicillins, but have no effect on other carboxy penicillins, ureido-penicillins, aztreonam, and carbapenems.

iii. Extended-spectrum b-lactamases (ESBL) inactivate majority of β-lactam antibiotics except carbapenems.

iv. Carbapenemases inactivate carbapenems and dependingon the enzyme, other types of β-lactam molecules as well.(26)

4.3.1.1 Introduction

Carbapenemases are a class of versatile β-lactamases, which have hydrolytic activity against the carbapenems and most of the other β-lactam antibiotics. They are also most resistant to hydrolysis by β lactamase inhibitors.By 1990's most classes of carbapenemases had been discovered and were thought to be specific for the species in which they were identified and chromosomally mediated. But the discovery of similar genes in other species of bacteria led to need for classification and study of these enzymes (6).

4.3.1.2 Classification schemes

The classification of β-lactamase enzymes are based on either functional characteristics or thestructure of the enzymes. The Amblerclassification is based on the structure i.e.protein sequence. In this classification on the basis of amino acid homology the enzymes are classified into four classes A, B, C, and D. Classes A, C,and D include enzymes that have serine at its active site and hydrolyze their substratesby formingan acyl enzyme. ClassB β-lactamases utilize at least one zinc ion at the active site to hydrolyse β-lactams and hence named

metallo-enzymes. The structural classification was simple and less controversial but lacked the details of the enzymatic activity of the enzymes such as hydrolytic and inhibition properties(31). The functional classification was first proposed by Bush in 1988 and many additions and modifications were made as the new enzymes with their differing functional profile were discovered.

Table 4.3. Classifiction of β- lactamase enzymes

Bush-Jacoby group (2009)	Molecular class (subclass)	Substrate(s)	Attributes	Representative enzymes
1	C	Cephalosporins	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolysis of cephamycins	ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	CMY-37
2a	A	Penicillins	Greater hydrolysis of benzylpenicillin than cephalosporins	Staphylococcal penicillinases
2b	A	Penicillins, early cephalosporins	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1,2 ,SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino- β-lactams (cefotaxime, ceftazidime, ceftriaxone,	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1

			cefepime, aztreonam)	
2br	A	Penicillins	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino- β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	A	Carbenicillin	Increased hydrolysis of carbenicillin	PSE-1
2ce	A	Carbenicillin, cefepime	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	D	Cloxacillin	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Hydrolyzes cloxacillin or oxacillin and oxyimino- β-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Increased	KPC-2, IMI-1,

			hydrolysis ofcarbapenems, oxyimino-β-lactams, cephamycins	SME-1
3a	B	Carbapenems	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1
3b	B	Carbapenems	Preferential hydrolysis of carbapenems	CphA, Sfh-1

4.3.1.3Molecular Class-A β- lactamases

Carbapenemases belonging to class A of molecular classification and group 2f of the functional classification were discovered over 20 years ago and now have been sporadically isolated from various clinical samples. These β--lactamases have been found in various genera such as*Serratia spp*,*Enterobacter spp*and *Klebsiella spp*. The enzymes that fall in this group are KPC *(K. pneumoniae* carbapenemase), GES (Guiana extended-spectrum β- lactamases, Nmc-A (non- metallo-carbapenemase-A)/ IMI (imipenem-resistant), SME *(Serratia marcescens* enzyme), SFC (serratia fonticola carbapenemase), and BIC β--lactamases (21).They occur as sporadic isolates or as outbreaks in hospitals. The chromosomally encoded enzymes of this class are SME, NMC, and IMI. The antibiotic profile of bacteria expressing these chromosomal β-lactamases belonging to group 2f is distinct. They exhibit resistance to carbapenems and susceptibility to extended spectrum cephalosporins.These enzymes confer the

bacteria carrying them with reduced susceptibility to imipenem, the MICs(minimum inhibitory concentration) for these enzymes can have a variable range from mildly high to completely resistant. These β-lactamases, therefore, may be difficult to recognize on routine susceptibility testing by disk diffusion methods (6). KPC and GES are the plasmid encoded enzymes in this group. Two features that separate the KPC carbapenemases from the other carbapenemases in the functional group 2f. First, that these enzymes are found on plasmids that are transferrable and secondlythathydrolyse cephalosporins such as cefotaxime which are classified as amino-thiazoleoxime cephalosporins. The KPC β-lactamases have largely reported from Klebsiella spp.they have also been reported from *Enterobacter* spp. and *Salmonella*spp.

The KPC family has the highest potential for spread among the group 2f enzymes because of its location on plasmids, found in *Klebsiella spp* which are bacteria known for their ability transfer resistance plasmids to other bacteria(6)

The GES is a not so frequently found family of carbapenemases. It was first identified from *E. cloacae* in Greece. They have been reported from other parts of the world subsequently. They have a wide spectrum of hydrolytic activity similar to ESBL's from extended spectrum penicillins to carbapenems sparing the monobactums.(6)

KPC is the most commonly isolated clinically all the other enzymes of this class are rare.(32)

4.3.1.4 Class B metallo- β—lactamases

Metallo β--lactamases are characterized by their distinct functional properties: ability to hydrolyze carbapenems, resistance to β- lactamase inhibitors, susceptibility to inhibition by ion chelators such as EDTA(33). Their spectrum of substrates hydrolysed include carbapenems, cephalosporins and penicillins. These enzymes lack hydrolytic activity against aztreonam. The mechanism of action is based on interaction of the β- lactam ring with the zinc present at their active site. The zinc ions present at their active site are vital for the nucleophilic hydrolysis of the β--lactam ring. This property makes them susceptible to metal ion chelators such as EDTA(6). Three lineages of the metallo β- lactamases have been suggested based on phylogenetic analysis B1,B2 and B3 (34). This classification scheme is based on the on the placement of the zinc organising residues and comprises of both chromosomal and plasmid encoded enzymes. The subgroup B1 includes chromosomally encoded β-lactamases enzymes and plasmid borne acquired enzymes like IMP,VIM,NDM,SPM. These variants have been found in *K. pneumoniae* and other genera in the family *Enterobacteriaceae*. They are grouped together as they possess the zinc coordinating residues histidine and cysteine. Class B2 comprises of those enzymes which contain asparagine instead of histidine at the zinc binding motif and are derived from *Serratia fonticola* and *Aeromonas spp*. Class B3 has only one enzyme L1 which is represented as a tetramer in contrast to the other enzymes (35). IMP-1 was the first among these carbapenemase to be identified from Japan in 1991from Serratia marcescens and was recognized as a source of resistance which is acquired. VIM-1 was described in 1997 froman isolate of *P. aeruginosa*Verona in Italy.IMP amd VIM share less than 30%

identity in the amino acids they have similar spectrum profiles. IMP and VIM producing *K. pneumoniae* have been found to have MIC for carbapenemases ranging from 4 to 32 μg/ml. The variability in the MIC's may pose a challenge in their identification in the laboratory (36). NDM enzyme was identified in 2008 and were the latest enzymes to be discovered. These enzymes have now been reported from different countries worldwide. They are known to cause hospital acquired and community acquired infections, and also in urban contaminated water from the Indian subcontinent. The predominant type is NDM-1, but many minor variants have been isolated. NDM enzymes are present principally in *Enterobacteriaceae*, in non-fermenting gram negative bacilli and family*Vibrionaceae*. The $bla_{\text{NDM-1}}$ genetransfer has resulted in the widespread dissemination of the gene.The NDM carrying bacteria are usually resistant to almost all antibiotics, and their detection and surveillance are vital.(37)

4.3.1.5 Class D serine-carbapenemases: the OXA β—lactamases

In the 1970's and early 1980's Oxacillin-hydrolyzing (OXA) β-lactamase enzymes were the most prevalent enzymes of the plasmid encoded enzyme families. They were initially identified in the non-fermenters and later in the family *Enterobacteriaceae*and were placed in a different class from the other serine β-lactamases. They were β-lactamases capable of hydrolysing penicillins, cloxacillin, oxacillin and were poorly inhibited by clavulanic acid and EDTA(6). Hence they were given the name oxacillinases and the prefix OXA. They were placed in the group 2d of the Bush-Jacoby functional classification. The class D β- lactamases have been idientified

mainly as plasmid encoded enzymes in contrast to class A and C. The early oxacillinases were penicillinases until an ESBL oxacillinase OXA-11 was isolated from *P. aeruginosa,* as a result of mutation. Several ESBL OXA enzymes were described following this. The OXA -23 was the first carbapenem resistant oxacillinase described which had high imipenem MIC (38). Many subgroups of the OXA carbapenemases have been described based on the amino acid homologies as abridged in table 4.4.

The OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, OXA-134a- like, OXA-143-like, OXA-213, OXA-214-like, OXA-211-like, OXA-229-like, OXA-235-like carbapenemase have been identified and are restricted to *Acinetobacter spp* where as a novel oxacillinase hydrolysing carbapenems was isolated from a *Klebsiella pneumoniae* isolate from Istanbul in Turkey. This enzyme variant was named OXA-48. The OXA-48 and its variants have now been isolated from different parts of the world in *Enterobacteriacae* and also *A. baumannii* (38). The enzyme kinetics of the OXA-48 enzyme demonstrated that they have low hydrolytic activity against carbapenems, with more potent activity against imipenem than against meropenem. They are not inhibited by β- lactamase inhibitors like clavulanic acid, tazobactum and sulbactam (39). 10 more OXA-48 variants have been recognised which include OXA-162, OXA-163, OXA-181, and OXA-232. OXA-163 has a poor hydrolytic activity against carbapenems but has ability to hydrolyze aztreonam and ceftazidime, which is not observed with OXA-48. The OXA-181 and OXA-232 enzymes seem to be approximately like OXA-48 in their activity (38).

Table4.4 Carbapenemase OXAβ- lactamases and their variants

Group of OXA carbapenemases	Enzymes in the group
OXA-23-like	OXA-23, OXA-27, OXA-49, OXA-73, OXA-102, OXA-103, OXA-105, OXA-133, OXA-134, OXA-146, OXA-165–OXA-171, OXA-225, OXA-239
OXA-40-like	OXA-40, OXA-25, OXA-26, OXA-72, OXA-139, OXA-160, OXA-207
OXA-51-like	OXA-51, OXA-64–OXA-71, OXA-75–OXA-80, OXA-82– OXA-84, OXA-86,OXA-95, OXA-98–OXA-100, OXA-104, OXA-106–OXA-113, OXA-115–OXA-117, OXA-120–OXA-128, OXA-130–OXA-132, OXA-138, OXA-144, OXA-148–OXA-150, OXA-172–OXA-180, OXA-194–OXA-197, OXA-200–OXA-203, OXA-206, OXA-208, OXA-216, OXA-217, OXA-219, OXA-223, OXA-241, OXA-242, OXA-248–OXA-250, OXA-254
OXA-58-like	OXA-58, OXA-96, OXA-97, OXA-164
OXA-134a- like	OXA-134a, OXA-186–OXA-191
OXA-143-like	OXA-143, OXA-182, OXA-231, OXA-253, OXA-255
OXA-213	OXA-213
OXA-214-like	OXA-214, OXA-215
OXA-211-like	OXA-211, OXA-212, OXA-309
OXA-229-like	OXA-228,OXA-230, OXA-257
OXA-235-like	OXA-235,OXA-237, OXA-278
OXA-48-like	OXA-48, OXA-48b, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247

4.3.2 Non carbapenemase mediated mechanisms

4.3.2.1 Loss of porin channels

The Gram-negative bacteria are protected from external toxic agents such as heavy metals and detergents by the outer membrane which is hydrophobic in nature. The outer membrane contains certain trans- membrane proteins, called porins. Porins act as hydrophilic channels for the entry of vitalnutrients and other compounds, as well as antibiotics. (40). The passage of molecules through porins is size dependent; smaller molecules pass through easily than the larger ones(41). The porin channels are of various types out of which the outer membrane porins (Omp) are significant in *Enterobacteriaceae*. The porin channels involved in the uptake of antibiotics in *Enterobacteriaceae* belong to the family OmpF or OmpC. (42)

A number of studies have reported an association between modifications in the porin channel type in antibiotic-resistant bacterialisolates. *Enterobacteriaceae* can show analteration in the type of porin channels they express, low level of porin expression orthe existence of a mutated porin channel. (42,43)β-lactam antibiotics are the most commonly affected class of antibiotics by porin changes as porin channel are a part of the entry pathway of these drugs . Therefore, any alterations in the number oractivity of porin channelscan have an influence on antibioticresistance.

In *Klebsiella pneumoniae*, OmpK35 and OmpK36 are the major non-specific porins and in *Escherichia coli* OmpC, OmpF are the identified porins which help in the transportation of carbapenems into the bacterial cell. (44,45)

Resistance mediated by porin loss is related to the molecular size of the carbapenem. These strains develop high level resistance to the larger molecule ertapenem which

depend on porins for its entry. On the contrary, low level resistance or reduced susceptibility is observed with the smaller imipenem and meropenem(27)(23). Presence of ESBL and Amp C enzymes along with defects in outer membrane permeability can be responsible for carbapenem resistance in gram negative bacilli(26).

4.3.2.2 Efflux pumps

Efflux pumps are complex proteins present in the bacterial cell envelope which actively pump out substances from the cell to the exterior. These pumps play a dual role in the bacterial physiology by excreting the poorly diffusible metabolites produced endogenously and by extruding out the harmful exogenous substances which diffuse in from the environment(47)(48). Hence administration of antibiotics have probably exerted a selective pressure for over expression of efflux pumps resulting in a non-specific mechanism of resistance(49).

Efflux pumps function either by primary or secondary active transport. The former acquires energy from ATP hydrolysis while the latter is driven by ion gradients. There are many families of efflux pumps with numerous members under each family, of which the members of the Resistance Nodulation Division (RND) super family contribute to antimicrobial resistance. All the pumps of the RND super family are secondary active transporters and are exclusively found in gram-negative bacteria. The notable member responsible for efflux of β--lactams in the *Enterobacteriaceae* is the AcrAB–TolC pump(50). Usually, strains resistant to carbapenems due to hyper-functioning efflux pumps also produce ESBLs(51).

Numerous natural and synthetic molecules have been tested for inhibition of efflux pumps so that they may be used *in vivo* for therapy but due to concerns about their efficacy and safety, none has been approved yet(42,50,52). Two compounds namely carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and phenylalanine arginine β-naphthylamide (PABN) are currently used for the *in vitro* detection of bacterial efflux pumps. Both these chemicals are broad spectrum efflux pump inhibitors (EPI) with different mechanisms of action. CCCP inhibits efflux pump activity by abolishing the ion gradient while PABN acts as a competitive inhibitor(53).

Apart from carbapenem resistance, efflux pumps also contribute to resistance to fluoroquinolones and other antimicrobials, antiseptics and detergents (50,54). Efflux pumps have also been implicated in the increased resistance of biofilms to antimicrobials (44) .It is also presumed that the AcrAB efflux pump is a virulence factor which enables *Klebsiella pneumoniae* to resist the innate defences of the lung and facilitate the onset of pneumonia(55).

4.4 Methods of detection of carbapenemases in *Enterobacteriaecae*

The detection of mechanism of resistance to carbapenems is of value in infection control and public health practices and epidemiological investigations. There are various techniques available for detection of carbapenemase production by *Enterobacteriacae*. They can be broadly classified into phenotypic tests and genotypic tests. Most phenotypic tests use the principle of inhibition by specific inhibitors, where the susceptibility to a carbapenemantibiotic is regained when tested along with inhibitors specific for each Ambler class of carbapenemase. Whereas the

genotypic tests directly detect the genes encoding for a specific type of carbapenemase enzyme.

4.4.1 Phenotypic methods

4.4.1.1 Disk diffusion testing with carbapenems

CLSI(The Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommend disk diffusion testing of the clinical isolateswith carbapenems such as meropenem, ertapenem, imipenem and doripenem as a screening test forcarbapenem resistance. Ertapenem disk diffusion is the most sensitive method but has less specificity. Meropenem disk diffusion has been found to have the best balance between sensitivity and specificity.

The breakpoints for carbapenem antibiotics for *Enterobacteriaceae* have been optimized to detect all clinically important resistance mechanisms of resistance, including the majority of the carbapenemases. (56)

Table 4.5 CLSI and EUCAST 2014 Breakpoints for disk diffusion testing

Carbapenem	**Disk strength**	**CLSI zone diameter interpretative criteria (mm)**			**EUCAST zone diameter interpretative criteria (mm)**	
		Susceptible	Intermediate	Resistant	Susceptible	Resistant
Doripenem	10µg	≥23	20-22	≤19	≥24	<21
Ertapenem	10µg	≥22	19-21	≤18	≥25	<22
Imipenem	10µg	≥23	20-22	≤19	≥22	<16
Meropenem	10µg	≥23	20-22	≤19	≥22	<16

4.4.1.2 Minimum inhibitory concentration for carbapenems

The minimum inhibitory concentration (MIC) of the isolates can be determined by macrobroth, microbroth dilution techniques or E-test method and can be interpreted using the standard guidelines as screening test to detect carbapenem resistance.

Table 4.6CLSI and EUCAST 2014 Breakpoints for MIC testing

Carbapenem	**CLSI MIC interpretative criteria (µg/ml)**			**EUCAST MIC interpretative criteria (mg/L)**	
	Susceptible	Intermediate	Resistant	Susceptible	Resistant
Doripenem	≤1	2	≥4	≤1	>2
Ertapenem	≤0.5	1	≥4	≤0.5	>1
Imipenem	≤1	2	≥4	≤2	>8
Meropenem	≤1	2	≥4	≤2	>8

In addition to manual methods mentioned above, many automated instruments are available which are capable of determining and interpreting the MIC of the test organism to a panel of antimicrobials. The noteworthy automated systems include Phoenix (Becton Dickinson) Vitek 2 (bioMérieux) and MicroScan (Siemens). Recent studies have shown manual methods to be more accurate than automated methods.(57)

4.4.1.3 Phenotypic confirmatory test- Modified hodge test

Modified hodge test (MHT) is recommended as a phenotypic confirmatory test by CLSI to detect the production of carbapenemase enzymes produced by the isolates. Carbapenemase production is detected by the MHT when the test isolate produces the

enzyme and allows growth of a carbapenem susceptible strain (*E.coli* ATCC 25922) towards a carbapenem disk. The result is a characteristic cloverleaf-like indentation. A positive MHT indicates that this isolate is producing a carbapenemase. A negative MHT indicates that this isolate is not producing a carbapenemase(58).

The advantages of modified hodge test are that it is simple to perform, many isolates can be tested on the same plate and carbapenems of different classes can be detected by a single test (59). (fig.4.2)

The limitations arethe class of carbapenemase cannot be determined by the results of the MHT, false positive results have been reported with AmpC β- lactamases. The modified hodge test has been observed to detect Class A and D carbapenemases, but has a lower sensitivity for NDM producing organisms(46, 47,48). The results are also ready 24 hours after the isolate has been identified, thus it is considered time consuming.

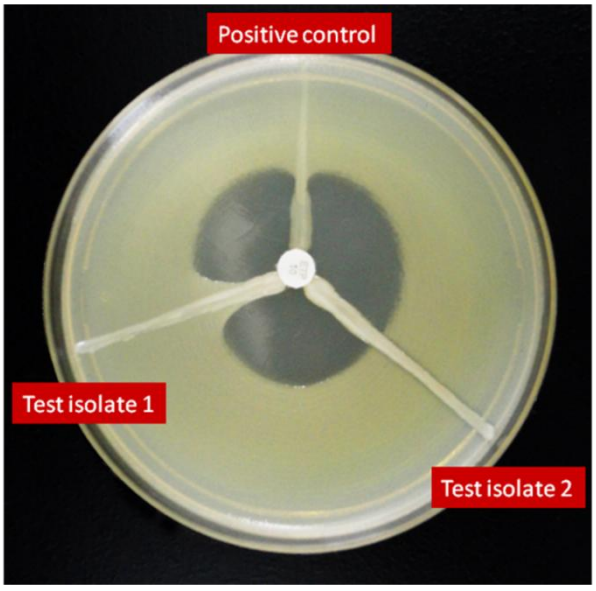


Fig.4.2 Modified hodge test for carbapenemase detection

4.4.1.4 Phenotypic detection using specific inhibitors

Specific inhibitory substances can be used to differntiate between the different groups of carbapenemases. Phenyl boronic acid inhibits class A carbapenemases like KPC without inhibiting the growth of the organism. EDTA and dipicolinic acid inhibit the metallo β- lactamases. Dipicoloinic acid has been found to be more specific than EDTA when synergy with carbapenem are used for identification of Mβ-L. Currently no inhibitors are available for detection of OXA-48-like enzymes. High-level resistance to temocillin (MIC >32 mg/L) has been proposed as a phenotypic marker for OXA-48-like carbapenemase producers(60,61). As these markers are not specific for OXA carbapenemases genotypic confirmation is required.

These substances are available commercially as disks of inhibitor-carbapenem combination or tablets and the E-test (Rosco NeoSensitabs^{TM} – Carbapenemases/Metallo-β--Lactamase Confirmative Identification pack). Table 4.7 summarizes the detection of β-lactamases using specific inhibitors.

Table 4.7 Mechanism of resistance to carbapenems using specific inhibitor substances

Mechanism	Synergy with meropenem disc			Temocillin
	DPA/EDTA	APBA	CLX	MIC >32µg/ml
KPC	-	≥ 4	-	-
MBL	≥ 5	-	-	-
OXA-48 like	-	-	-	Yes
AmpC+ porin loss	-	≥ 4	≥ 5	-
ESBL+ porin loss	-	-	-	No

Abbrevations used in the table

APBA: aminophenylboronic acid, DPA: dipicolinic acid, EDTA: ethylenediaminetetraacetic acid, CLX= cloxacillin

4.4.1.5CarbaNP test

The CarbaNP test is a new phenotypic test for the detetction of carbapenemase production by *Enterobacteriaecae* and *Pseudomonas spp*. The principle of the test is in vitro hydrolysis of the imipenem ring by the carbapenemase produced by the bacteria to form an acidic compound which lowers the pH. The change in pH is detected by change of colour of the indicator phenol red from red to yellow.

The bacterial isolate to be tested is incubated in a bacterial cell lysis solution. Following this the supernatant is mixed with imipenem, phenol red and zinc sulphate.

The bacteria producing carbapenemase will turn the colour of the solution from red to yellow.

This test can be used for rapid screening (<2hrs) of carbapenemase mediated resistance in *Enterobacteriacae* and *Pseudomonas spp.* The sensitivity and specificity of this test were found to be 100% (62). Tijet *et al.* in 2013 evaluated the test and found that the specificity was 100% but the sensitivity was 72.5% as compared to 100% as published eariler. They also found the sensitivities to be lower for OXA 48 like producers and isolates carrying GES-5 and SME-1 producers. In addition they had false negative results with mucoid strians (63).

Table 4.8 summarizes the studies evaluationg CarbaNP test.

Table 4.8 Sensitivity and specificity of the CarbaNP test

Study	No. of isolates	Compared with	Sensitivity (%)	Specificity (%)	Reference
Nordman P *et al.*	162	-	100	100	(62)
Vasoo *et al.*	271	Modified hodge test	100	100	(64)
Knox *et al.*	105	PCR,MALDI TOF	87	100	(65)
Huang *et al.*	356	Rosco Rapid CARB screen kit	97	100	(66)
Tijet *et al.*	244	Modified hodge test	72.5	100	(63)

CarbNP is a rapid test, technically less demanding, inxepensive as compared to molecular test and also has a good sensitivity and specificity. Dortet *et al.* evaluated a slightly modified protocol to use this test on positive spiked blood culture samples. With this modification the test can be used directly on samples and the detection of carbapenemases can be done within 3-5 hours(67).

4.4.1.6 Chromogenic media

Many chromogenic media are available for the identification and isolation of carbapenem resistane *Enterobacteriacae.* Brilliance CRE (Oxoid, Thermofisher Scientific, Illkirch, France) CHROMagar KPC (CHROMagar, Paris, France), SUPERCARBA medium and chromID OXA-48 (bioMérieux) are some of the assessed and used media.

CHROMagar KPC contains chromogenic media which indicate growth of *Enterobacteriaceae*. It was useful in detecting high level resistance to carbapenems and also was found to have a sensitivity of 40.3% and specificity of 85.5%. among the various classes of carbapenemases it had the lowest sensitivity for OXA-48 producers of only 13.6% (68).

Brilliance CRE also contains chromogenic media to differentiate *E.coli* and other *Enterobacteriaceae*. It was found to have a good sensitivity of 94% but lacked specificity 71% as growth ESBL and AmpC producers was also seen (69). Nordmann *et al.* reported a sensitivity and specificity of 60.7% and 57.1%, respectively. The sensitivity to individual Ambler class A,B,C enzymes was found to be 85%, 78.4% and 69.8% respectively (68).

SUPERCARBA medium is Drigalski agar-based medium to which ertapenem, cloxacillin, and zinc sulphate were added to improve detection of low level resistance to carbapenems and inhibit growth of non-carbapenemase producers. The sensitivity of detection of carbapenemase produceing organisms by the SUPERCARBA medium (96.5%) was greater

than those of the chromogenic media discussed above. Specificity of the medium was 60% which lower than CRE Brilliance agar. The sensitivity of this medium for detection of Class A and D carbapenemases was found to be 100% (68,70).

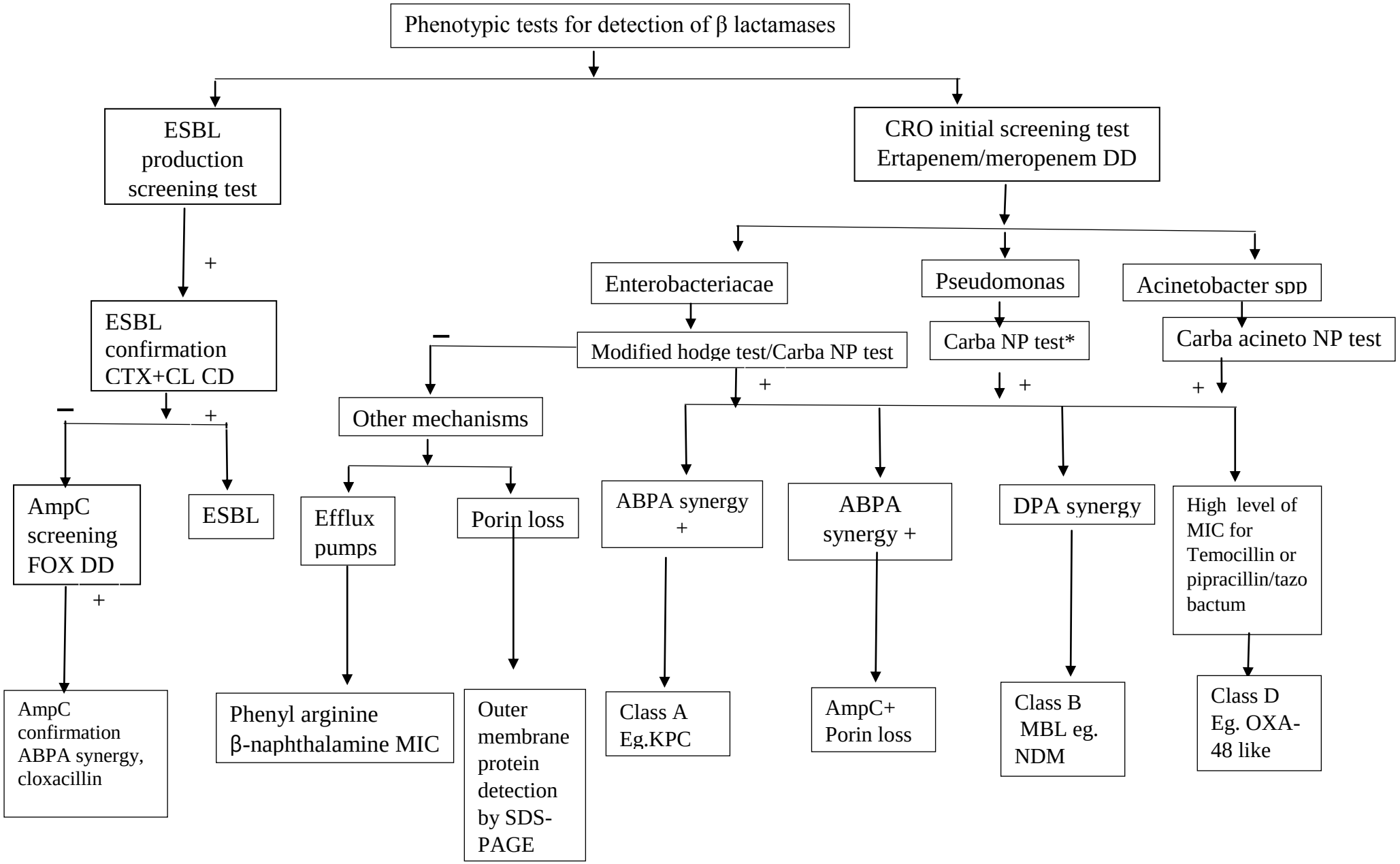
chromID OXA-48 has been found to have a high sensitivity for detection of OXA like carbapenemases 91% and also has a higher specificity of 100% as compared to the 60% of SUPERCARBA (71).

Chromogenic media chromID® CARBA has also been found to have a sensitivity of 96.5% and a specificity of 91-100% when used for screening of rectal swabs for carbapenemase producing *Enterobacteriaceae*(72).

Table 4.9 Summary of phenotypic tests for detection of carbapenem resistant *Enterobacteriaceae*

Phenotypic methods	Carbapenemases	Result	Comments	Reference
Modified Hodge Test (MHT)	KPC, OXA-48	Sensitivity:58% Specificity: 93%	Poorly detects NDM, VIM & IMP Cannot differentiate class of carbapenemase False positive results with CTX-M ESBL or increase amounts of AmpC producers Difficult to interpret with bacteriocin production	(73)
RoscoDiagnostica Neo-sensitabs	KPC & NDM	Sensitivity: 80% Specificity: 93%	Poorly detects VIM, IMP & OXA -48 Reliable phenotypic test for detection & categorization	(73)
RoscoDiagnostica (KPC/MBL & OXA-48 confirm kit 98015)	KPC, MBL & OXA-48	Sensitivity: Class A – 95% Class B – 90% OXA-48 : 100% Specificity: 96% - 100%	In vitro identification of bacteria producing carbapenemases and oxacillinase by the agar tablet/disc diffusion method Simplistic method of detecting bacteria producing KPC, MBL and OXA-48.	**(74)**
Combined disc test or disc enhancement test	Differentiates KPC from NDM	KPC Sensitivity:100 % Specificity:98.8 % NDM Sensitivity :100% Specificity: 100%	Early differentiation of carbapenemase Good sensitivity & specificity	(75)
CHROMag	KPC,	Sensitivity:40.3	High levels of carbapenem	(68)

ar KPC	IMP, VIM, NDM, OXA-48	% Specificity:85.5 %	resistance is detected Lacks sensitivity	
CRE Brilliance	KPC, NDM, GIM,VIM, OXA -48	Sensitivity: 94% Specificity: 71%	Often fails to detect OXA -48 Low specificity	(69)
SUPERCARBA	KPC, VIM, IMP, NDM-1, OXA-48, OXA-181	Sensitivity:95.6 % Specificity:82.2 %	Detects low level resistance Inhibits non-carbapenemase producers	(68)
chromID OXA-48	OXA-48 OXA-48 variants with carbapene mase properties (OXA-162, OXA-181, OXA-204 & OXA-232)	Sensitivity : 91.2% - 96.5% Specificity: 100%	Lowest limit of detection ranges from 1x 10^1 to 1x 10^2 CFU/plate chromID OXA-48 is as sensitive for detection of OXA-48 producers as SUPERCARBA medium, but with higher spwcificity	(72)
chromID CARBA	OXA-48	Sensitivity : 30% Specificity : 67.5%	Weak sensitivity for detection of OXA-48 producers Powerful tool for detection of all other classes of CPE	(71)
Carba NP	KPC, NMC-A, SME, GES, IMI, NDM, VIM, IMP, OXA-48	Sensitivity: 97.9% Specificity: 100%	Enzymatic test Rapid Reproducible Inexpensive	(67)



* Alternatives: CHROM agar KPC, CRE Brilliance agar, Chrom ID ESBL, ChromID CARBA

CPD-cefpodoxime, CTX-cefotaxime, CZD- ceftazidime, CL- clavulanic acid, FOX- cefoxitin, DD-disk diffusion, MIC minimum inhibitory concentration, ABPA Aminophenyl boronic acid, DPA dipicolinic acid

4.4.2 Molecular methods

Molecular methods used for the detection of carbapenem genes are reference tests for the identification or the resistance mechanisms. These tests are based on identification of the genes encoding the enzymes. Most of the methods are PCR based. The methods are rapid and accurate but require costly equipment and trained personnel. The genes that can be detected by these methods are also predefined and new genes may not be identified by molecular techniques (76)(13) .

4.4.2.1 PCR based methods

4.4.2.1.1 Conventional uniplex amd multiplex PCR

PCR is considered a rapid approach to determine the β- lactamase present in a suspected carbapenem resistantisolate. Several uniplex PCR assays targeting a single carbapenemase gene type have been used previously. Uniplex PCRs donot prove to be cost efficient as the same isolate has to be run for many different genes, a co-existence of genes cannot be detected in a single reaction(77).

Asseveral clinical isolates harbouring more than oneβ-lactamase gene are encountered and the enzymes exhibit high varietyand versatility, multiplex PCR techniques are being recommended for epidemiological surveys (77). Ellington *et al.* in 2007 established a multiplex PCR assay for the simultaneously detecting five different families of carbapenemase encoding genes. IMP, VIM, SPM,SIM and GIM of acquired metallo-β- lactamase genes in a single reaction. The multiplex PCR assay could reliably detect and differentiatebetween all the genes tested (13)(78).

Later, as the other classes of carbapenemases such as the Class A KPC, NDM belonging to class B and Class D OXA-48 like enzymes began to emerge an updated multiplex PCR protocol was described by Poirel *et al*. This assay employed three multiplex PCR reactions and was aimed at detecting the evolving carbapenemase genes KPC, NDM-1 and OXA-48, in addition to the already discovered(79).

Dallenne *et al.* designed a multiplex PCR for detection of common β- lactamases in gram negative bacilli which caused resistance to third generation cephalosporins and carbapenems. The protocol included six multiplex PCR reactions for identification of common ESBL, AmpC and Class A,B and D carbapenemase genes (77).

The multiplex PCR assays had the advantage of detecting the most commonly prevalent genes and are less cumbersome than uniplex PCR's.

PCR/electrospray ionization–mass spectrometry (MS) technology is a system which has the capacity to detect multiple genes present in an isolate, it also detects single nucleotide polymorphisms. This assay has been used to characterize bla_{KPC} enzymes in the family *Enterobacteriaecae* (80).

4.4.2.1.2 LAMP

LAMP (Loop-mediated isothermal amplification) is an alternative method of DNA amplification with high specificity, efficiency and occurs under isothermal conditions. It employs a DNA polymerase and a set of four specially designed primers that recognise six DNA sequences on the target DNA. The final products of LAMP are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted

repeats of the target in the same strand which enable their simple, easy, selective detection by electrophoresis (81). The reaction results in the accumulation of 10^9 copies of target and simply requires a laboratory water bath or heating block to maintain a constant temperature of 60–65°C, making it particularly suited to resource poor settings. Detection of products is done by agarose gel electrophoresis, real-time monitoring in an inexpensive turbidometer or in the form of a colour change when SYBR Green I, a fluorescent dsDNA intercalating dye is used which detects turbidity or a pellet. Moreover, the LAMP assay is not affected by polymerase inhibitors (82). A LAMP assay for detection of NDM gene using eight primers has been standardized and has a sensitivity and specificity comparable to PCR (83).

4.4.2.1.3 Real-time PCR methods

The first multiplex real-time assay was developed by Mendes *et al.* to detect MBL genes encoding IMP, VIM, GIM,SIM, SPM enzymes. A melt curve step was donefollowing the real time PCR and amplicons melting peak analysis was used to identify the carbapenemase genes(84). Chen *et al.* developed a multiples PCR reaction which had 100% sensitivity and specificity for detection of bla_{KPC} gene in the ST258 clone of *K. pneumoniae*. The assay was a rapid and simple to perform test and demonstrated good sensitivity and specificity. It was found to be a useful test for ST258 *K. pneumoniae* surveillance in outbreak and epidemic settings (85). Recently a multiplex real time PCR has been developed for the rapid detection (3 hours) of bla_{VIM} ,bla_{KPC} , bla_{GES}, bla_{IMP}, bla_{NDM-1} andbla_{OXA-48} genes in *Enterobacteriaecae.* All the six carbapenemase genes looked for in the assay presented a different melting curve after

PCR amplification. The assay showed good reproducibility and was rapid for the identification of carbapenemase producing clinical isolates (86).

4.4.2.1.4 Microarrays

Microarray are DNA hybridization techniques that permit simultaneous detection of many sequences.Ulyashova *et al.* established a microarray technique which could identify GIM, KPC,OXA, IMP, VIM, SPM and SIM carbapenemases.The technique includes a number of steps, comprising DNA extraction from the sample and amplification of the gene present a multiplex PCR accompanied by concurrent labelling by biotin. The biotin labelled PCR product are hybridized with oligonucleotide probes which are immobilized on the surface of a nitrocellulose-based DNA microarray. The molecules of biotin thus get attached to the DNA duplexes are identified by using streptavidin-horseradish peroxidase as the conjugate. The microarray systems which are commercially available for the detection of ESBL's and carbapenemases have been found to have excellent sensitivity and specificity.

Microarray technology also proves useful as carbapenemases detection directly from blood samples has also been standardized. Sensitivity can be affected by the technique used for DNA extraction. Microarrays have not yet been standardized for other samples. The major limitation of microarray for routine diagnostics is its high cost and requirement of trained technicians. This technique may prove to be of great value for epidemiological investigations (13)(87).

4.4.3 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

The detection of carbapenemase production by the use of MALDI-TOF MS were standardized in 2011. In the assay, bacterial culture is mixed withcarbapenem solution (meropenem or ertapenem).It is then incubated at 35–37°C for 2–4 h, centrifuged and the supernatant is measured by MALDITOFMS. The degradation products and carbapenems are assessed for hydrolysis by mass spectrometry. The rate of false positivity is low in this technique. Some OXA 48 like enzyme producing strains and mucoid strains can give false negative results. The assay has been standardized for *Enterobacteriaceae* and had a sensitivity of 96.67% and a specificity of 97.87% (88).

4.4.4 Spectrophotometric assays

For the detection of enzymes by spectrophotometric method a bacterial crude extract is prepared and is mixed with a buffered solution of a carbapenem following which the hydrolysis of the β- lactam ring is estimated in UV spectra. The limitations of this technique is that it is labour intensive but has been proposed as a reference confirmatory method for carbapenemase production (89).

The molecular techniques are summarized in table 4.10

Table 4.10 Molecular techniques for identification of carbapenem resistance

Molecular methods	Carbapenemase	Result	Comments	Reference
Multiplex PCR	IMP, VIM, OXA-48, GIM, SPM, NDM, KPC, GES, PER	Sensitivity:100% Specificity:100%	Good sensitivity & specificity Expensive, requires a trained professional.	(77,78)
LAMP Assay	NDM-1	Greater sensitivity & specificity than PCR	Rapid, simple, cost – effective, Specific, Gel electrophoresis not required 1pg genomic DNA/tube – detection limit	(72)
Real time PCR with High resolution melting (HRM)	KPC	Sensitivity:100% Specificity :100%	Can differentiate between KPC-2 and KPC-3 by using HRM analysis curve Rapid and statistical differentiation of KPC variants	(85)(90)
DNA microarray (Check – MDR CT 102 array)	NDM, VIM, OXA-48, KPC, IMP	Sensitivity: 97% Specificity : 100%	Practical & Rapid tool Expensive	(91)
Matrix-assisted laser desorption ionization-time of flight (MALDI-TOFMS)	VIM, IMP, NDM-1 and KPC-2	Sensitivity:96.67 % Specificity:97.87 %	Quick, cheap tool for identification Lowest detection limit was found to be 50µM.	(88)

Check-KPC/ESBL microarray	ESBL producers (SHV, TEM, and CTX-M) KPC	Sensitivity & specificity -100%	Novel detection assay Potential to detect unlimited number of genes within one reaction Rapid and highly discriminatory	(92)

4.4.5 Detection of non-carbapenemase mechanisms

The non carbapenemase mediated mechanisms can be over production of ESBL or AmpC enzymes with or without porin loss/ overexpression of efflux pumps.

The loss of porins or occurrence of modified porins can be detected by phenotypic and molecular methods. The phenotypic method encompasses extraction of the outer membrane proteins by sonication from a pure broth culture followed by electrophoresis on polyacrylamide gels with sodium dodecyl sulphate. Bands of 37 to 40 kDa are encountered with the major porins OmpK35 and OmpK36. The variants OmpK36v forms a 35 kDa band and OmpK26 forms a 26 kDa band(93). Molecular identification of genes coding for porins and their variants can be done by PCR using specific primers(94,95).

The in vitro tests currently available for detection of efflux pump activity are EPI based and non-EPI based methods. The EPI based methods comprise of performing the test in two sets, one with the substrate only and the other with both the substrate and the EPI. Various substrates used are antimicrobials such as carbapenems or fluoroquinolones, fluorescent compounds such as ethidium bromide or bis-benzimide and radioactive compounds such as [C^{14}] chloramphenicol. In tests using

antimicrobials, drop in the MIC of the antimicrobial in the presence of EPI denotes efflux pump activity(96).Intracellular accumulation of fluorescent or radioactive compound when tested along with the EPI signifies the efflux mediated resistance(97,98). Non EPI based methods include SDS-PAGE and mass spectrometry analysis of bacterial outer-membrane extracts(99).

4.4.6 Strategy for the detection of carbapenem resistance

CLSI and EUCAST have revised their disk diffusion and MIC breakpoints thus permitting better recognition of carbapenemase production. However the detection of carbapenem resistance based on only phenotypic methods is not sensitive. CLSI and EUCAST recommend the use of special tests for detection of carbapenemase mediated mechanism only for hospital infection control and epidemiological investigations. Nordmann *et al.* disagree with the guidelines and recommend special test is suspected isolates as carbapenemase producing bacteria can show *in vitro* susceptibility, but the therapeutic response to carbapenems in such patients is poor (89). They also recommend screening for carriers and colonizers to prevent the spread of carbapenem resistant bacteria in the hospital environment. (100).The flowchart for detection proposed is as follows (fig 4.3)

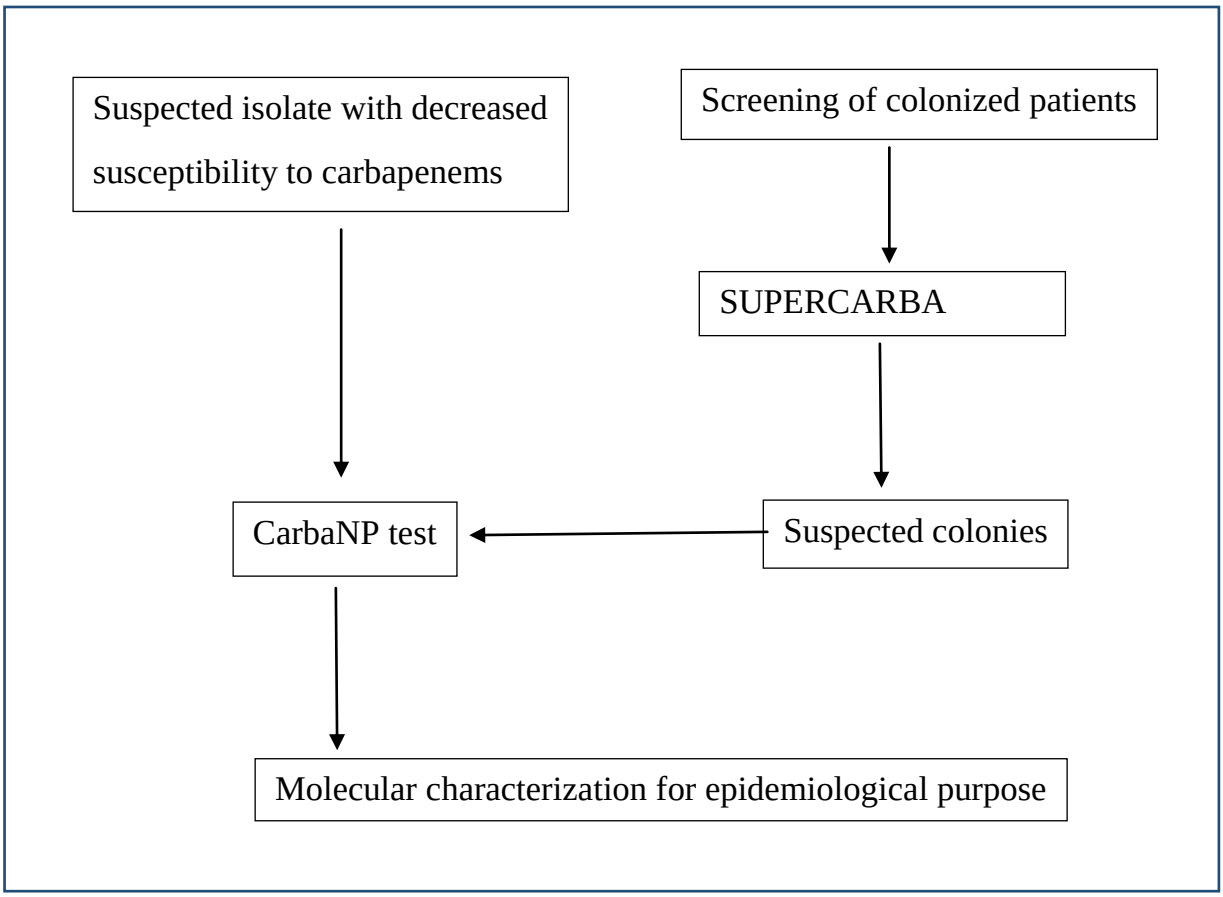


Fig. 4.3 P**roposed flowchart for the detection of carbapenem resistant isolates**

4.5 Epidemiology of Carbapenemase resistant *Enterobacteriaceae*:

4.5.1 Global scenario:

Resistance to carbapenem group of drugs started to become a problem due to their increased use in treating infections caused by ESBL's. It has acquired larger dimensions in the present world due to spread across continents. Until 1990's the carbapenemase resistance was a rare entity restricted to sporadic cases in hospitalised settings(32). However, data collected over the past decade has suggested a drastic

increase in resistance rates across the world. The initial emergence of carbapenemases usually is geographically restricted to a particular country. However, due to the ability of intra and inter species transmission of drug resistant plasmids by conjugation leads to dissemination across the world. Each of the genes vary in their capability of horizontal transfer. KPC, NDM, OXA-48 are the three majorly reported carbapenemases belonging to the three classes of ß-lactamases. USA, Israel, Greece and Italy are reported to be the key reservoirs of KPC enzymes, NDM are predominant in the Indian subcontinent identified in both *E. coli* and *K. pneumoniae*. The reservoirs of OXA-48 are both *Klebsiella pneumoniae* and *E. coli* identified from North Africa and Turkey. Of these enzymes KPC producers arefrequentlyrecognized in nosocomial isolates, on the other hand NDM and OXA-48 producers can be acquired either nosocomially or from the community (101) The $bla_{\text{NDM-1}}$ gene coding for NDM-1 is highly mobile and prefers horizontal spread while the bla_{KPC} gene is known for its clonal spread in *Klebsiella pneumoniae*(32)(102). ST258 is the prodminant clone and its variants ST151 and ST 11 have also been identified(103). Individuals from areas where carbapenemases do not exist travel to endemic areas seeking medical care. Thus they are susceptible to either infection or colonisation of drug resistant organisms. This further leads to spread of these organisms to non-endemic countries when they travel back.

4.5.1.1 Class A carbapenemases

The first documented infection due to a carbapenemase producing organism was in 1990 in France. This was from an isolate of *Enterobacter cloacae* and identified as NmcA(32). NmcA belongs to the current Class A (Ambler classification) and is

transferred chromosomally across bacteria. However, presently these are not very commonly detected(104). Though there were reports which were published later following this, the first carbapenemases seems to have emerged in as early as 1982 in UK and USA(32). The enzyme responsible was SME-1 belonged to a patient from London. Subsequently SME-2 and SME-3 were identified in various parts of USA but remain restricted to sporadic cases(105). Another enzyme belonging to class A is IMI which were first detected in 1984 in USA(106). IMI-1 and IMI-2 have been isolated from environmental sources across USA(107). The most important among the class A enzymes are KPC (*Klebsiella pneumoniae* carbapenemase). They are predominantly plasmid mediated and carry genes conferring resistance to other classes of antimicrobials such as Aminoglycosides and fluoroquinolones(32)(6). KPC-1 was first isolated from North Carolina in 1996. Currently there are 12 subtypes spread over various regions of the world. Several of these have been identified in various parts of USA in tertiary care settings as well as from the community(108). The gene bla_{KPC} is carried on a transposon and transmitted across bacteria thus leading to outbreaks in hospitalised settings. This has been confirmed by studies done which show a similar subtype isolated from almost all samples during an outbreak(109). The gene has also been isolated from several European countries and Israel (96). Several studies done in the recent past from 2007 onwards in Greece suggest an ongoing epidemic of KPC-2 in hospitalised patients(108,110). In 2004, KPC was detected in China and then spread to South Korea and Taiwan as well. It further spread to Singapore where cases were reported in 2012(111). KPC has not only been isolated from isolates of *Klebsiella pneumoniae* but also from other members of *Enterobacteriaceae*. KPC happens to be

the most common carbapenemase across the world (fig4.4)(108). However, due to the slow clonal spread of KPC, the rapidly disseminating NDM-1 is expected to become the commonest carbapenemase in the coming years(102).

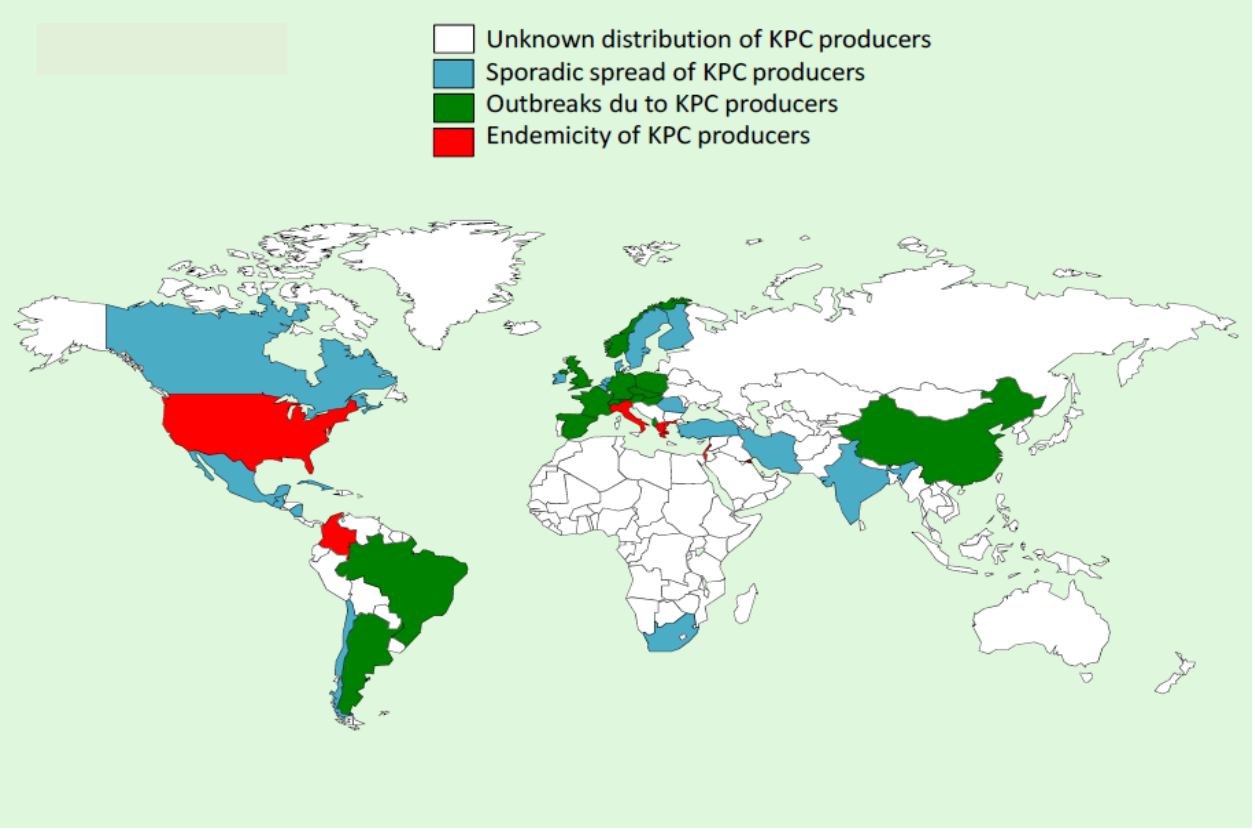


Fig. 4.4 Geographical distribution of KPC producing Enterobacterial isolates (Adapted from (101)**)**

4.5.1.2 Class B carbapenemases:

Class B metallo-β-lactamases are of two types; chromosomal and plasmid mediated. Plasmid mediated genes are mobile and hence are of growing concern due to rapid transmission.(fig.4.5) The first gene identified was IMP-1 from Japan in 1999. Later it spread to other European countries, Brazil and other parts of Asia. VIM is another enzyme isolated from Italy and now being increasingly identified from various parts of the world. Some of the metallo-β-lactamases are geographically

restricted such as SPM, GIM, KHM etc(32). Of importance is NDM which kindled interest in the world of microbiology for the first time in 2008. The gene was identified from a Swedish individual of Indian descent (112). Rapid dissemination of this gene across the world has been attributed to its ability to expressed in various gram negative bacilli and the mobile nature of the plasmid(32). Several studies followed the initial controversy regarding the origin of NDM. It has been suggested that NDM might have been circulating in Indian hospitals since 2006(113). Further studies suggested that it might have been imported to India from outside the continent. The gene might have been endemic in Balkans according to a studies conducted by several groups (114)(115)

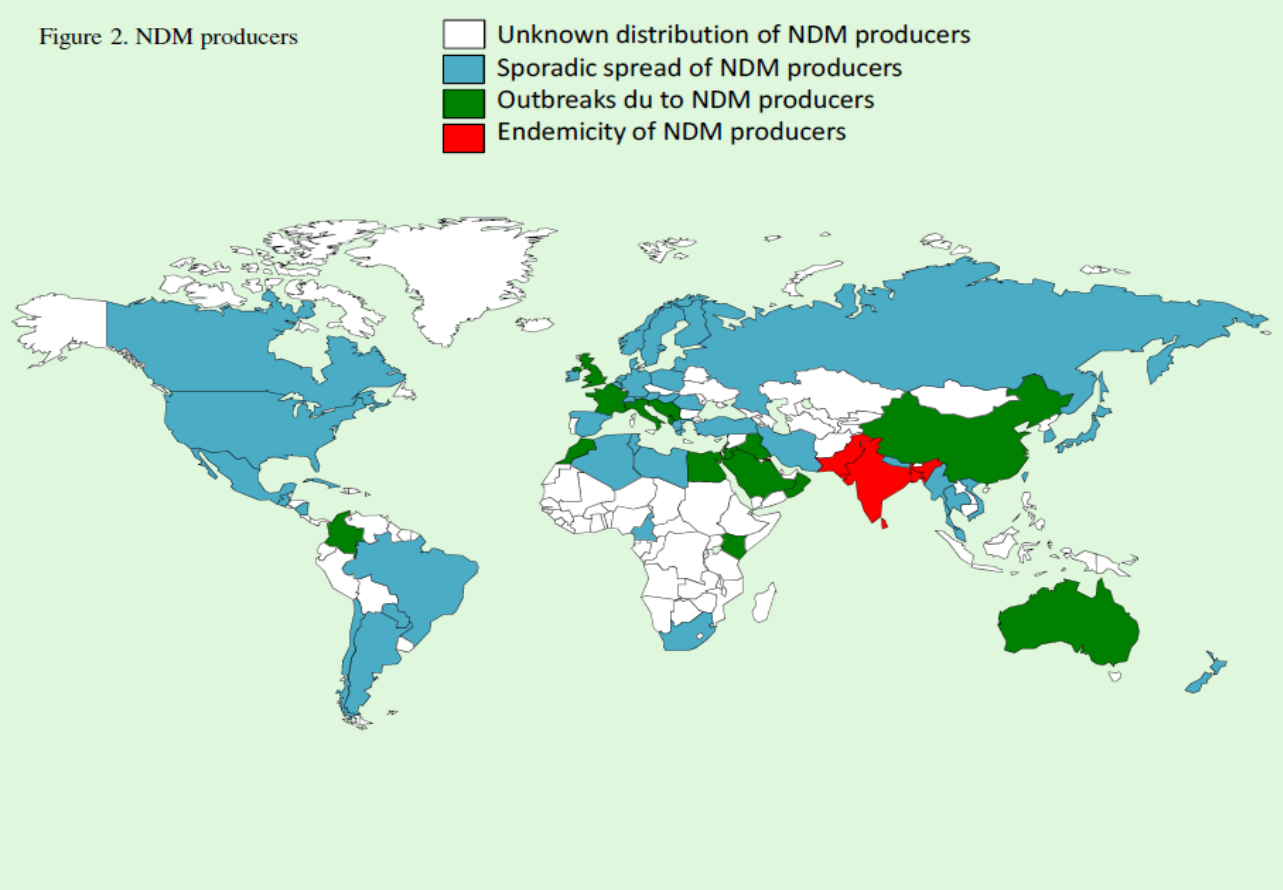


Fig. 4.5 Geographical distribution of OXA-48-like-producing enterobacterial isolates (adapted from (101)

4.5.1.3 Class D carbapenemases:

There are several varieties of class D β- lactamases (oxacillinases) of which only few can hydrolyze carbapenems(32). There are around 250 types. OXA -23 was the first oxacillinase discovered to have carbapenemase activity. This was isolated from Scotland in 1993 from *A. baumaanii* Most carbapenem hydrolyzing oxacillinases are found in *Acinetobacter spp*(32)(6). However OXA-48 is found in members of Enterobacteriaceae. This was recovered from Turkey for the first time in 2003 from a *Klebsiella pneumoniae* isolate. Initially it was thought to be restricted to the Middle Eastern countries. Currently, several outbreaks in various parts of the world have been reported. Hospitals across France, Spain, Netherlands, South Africa have reported OXA-48 in hospitalized patients(32).The existingdissemination of the *bla*OXA-48 gene is most probably associated with the spread of a single 62kbp self-transferrable IncL/M-type plasmid which does not transfer any othersupplementary resistance gene. OXA-48-type carbapenemases have been recognisedlargely from the Middle Eastcountries, North Africa, Turkey and India. These geographical areas constitute the most significant reservoirs. However, in the European countriesprevalence of OXA-48producers has been documented, some of them as hospital outbreaks (11). Fig.4.6 shows the geographical distribution of OXA 48 like enzyme producing *Enterobacteriaceae*.

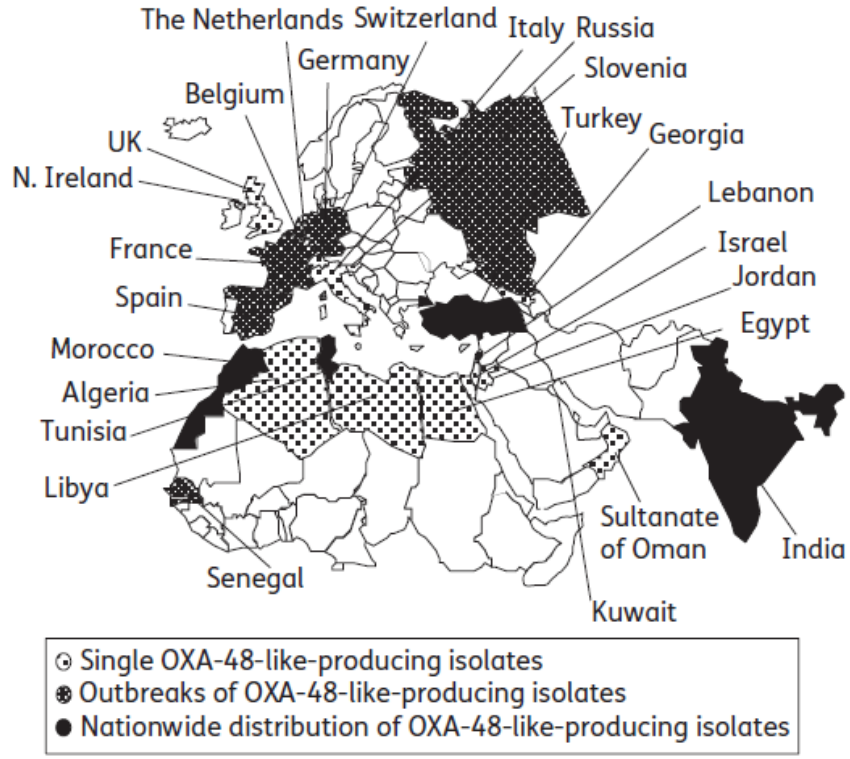


Fig. 4.6 Geographical distribution of OXA-48-like-producing enterobacterial isolates (Adapted from (11)

4.5.2 Indian scenario:

Data on CRE is being generated in India since the discovery of NDM which raised several controversies. Several studies from across the country have estimated the prevalence of CRE. Rates vary from 5.3% to as high as 51% (8,116)

NDM-1 was first identified in early 2008 from a Swedish patient with history of hospitalization and treatment in New Delhi, India. Phenotypic and phylogenetic studies confirmed the isolate as a novel MBL of Indian origin and hence given the name NDM-1. The emergence of this strain generated many political issues. Indian

authorities felt that it would have an unfavourable effect of tourism in India and suggested that the name be changed to PCM (Plasmid mediated metallo β-lactamase)(117). Several studies followed the initial controversy regarding the origin of NDM. It has been suggested that NDM might have been circulating in Indian hospitals since 2006(113). Further studies suggested that it might have been imported to India from outside the continent. The gene might have been endemic in Balkans according to a studies conducted by several groups(114)(115). NDM has gained such extensive patterns of spread due to the mobile plasmid. Although initially restricted to hospital based infections initially, it spreads to the community in no time due to inadequate sanitation facilities. Current data shows that NDM is the most common among carbapenemases in India.

A large surveillance on mechanism of antimicrobial resistance was conducted called the SENTRY Antimicrobial Surveillance programme in India. Results of the same were published in 2011 which showed that the most common gene isolated was NDM (38.4%) followed by OXA-181 (110-117). SMART also showed similar results. The other genes found were KPC and VIM(10). Following table shows results of various studies done identify the prevalence of carbapenem resistant *Enterobacteriaceae*.

Table 4.11: Epidemiology of Carbapenemase resistant *Enterobacteriaceae* in India

Region where study was done	Total no. of Enterobacterial isolates	Prevalence of CRE	Genes identified	Ref
Chennai	3521	4%	NDM 31%	(118)
Haryana	198	24%	NDM 55%	(118)
Mumbai	-	8%	NDM 91%	(119)
Jaipur	60	-	MBL 88%	(120)
New Delhi	423	51%		(121)
Guwahati	270	5.1%	NDM 100%	(122)
Kanchipuram	46	-	KPC 67.4 %	(123)
Lucknow	464	12.3%	NDM-1 77% NDM-5 3% NDM-6 14% NDM-7 6%	(124)
SENTRY New Delhi, Mumbai, Pune	1443	2.7%	NDM-1 57% OXA-181 39% VIM6 1 isolate OXA-181 &VIM6 1 isolate	(113)
Pune	300	15%	55.5% OXA 48 and NDM coexistence	(9)

Other than NDM, the genes which have been isolated in India include KPC, VIM, OXA. Data regarding OXA-48 is limited, but a few studies suggest the presence in the Indian subcontinent. A variant of OXA-48 which is OXA-181 has been reported but the magnitude is largely unknown (11). SENTRY study conducted in 2006- 2007 where samples were collected from major Indian cities such as New Delhi, Pune,

Mumbai showed that of the carbapenem resistant *Enterobacteriacae* isolated 57% were positive for *bla*NDM-1,

39% were positive for *bla*OXA-181, one isolate showed the presence of VIM6 and one isolate showed the coexistence of OXA-181 &VIM6 (113).

Only 1.8% of CRE isolates were positive for OXA-48 in a study done in Chennai and they concluded that it might not be a major mechanism of resistance(125). In a study done in 2014 in central India, the researchers found the co-existence of OXA-181 and NDM from an isolate. They also concluded that earlier the detection the better it is to limit the dissemination of drug resistant genes (9).

4.9 Treatment options

Bacteria harbouring the carbapenem resistance genes are extensive drug resistant organisms, owing to which limited treatment options are available. These infections also lead to frequent therapeutic failures. Colistin and tigecycline are the presently available options for treatment of infections due to carbapenem resistant organisms **(126)**. Colistin is available as colistimethate sodium which is the pro-drug administered through the intravenous route and has ca concentration dependent killing. The pharmacokinetic properties of this drug are variable, it has a poor distribution in various body sites. Its side effects include nephrotoxicity and usage of colistin leads to quick selection of resistant mutants. The above mentioned factors hinder colistin monotherapy**(127)**.

Tigecycline has a good *in vitro* activity against most carbapenem resistant organisms, but has variable *in vivo* activity when used therapeutically. It has a good tissue

penetration but a low plasma concentration and hence is not useful in treatment of blood stream infections. Breakthrough bacteremia and recurrence of infection are said to occur with tigecycline use. Data suggests that patients with severe illness treated with tigecycline as a single agent had slightly higher but significant mortality rates (128).

Fosfomycin is a derivative of phosphonic acid, an old antimicrobial agent with renewed interest has been evaluated for its efficacy against CRE. It has been observed to have a inconstant activity against diverse members of the *Enterobacteriaceae* family. Most strains *E. coli*, *Enterobacter spp*. and *Citrobacter spp*. are susceptible, while resistance is noted in over 50% of *Klebsiella spp*(129).

Other antimicrobials which can be used for treatment of rarely susceptible strains include aztreonam for MBLs without ESBLs, ceftazidime for OXA-48, aminoglycosides may be used for non KPC, non NDM-1 producing organisms and temocillin for non-carbapenemase mechanisms.(129,130,130)

Temocillin is a semi synthetic derivative of the drug ticarcillin. Data for its use in carbapenem resistant organisms is not available(128).

Therapeutic use of synergistic combination of antimicrobial agents hasbeen shown to have higher success rate than monotherapy with any agent.Studies which compare retrospective data favour combination therapy over single agent therapy, as the absolute difference in mortality ranges from20.2% to 46.7%(131). Various combinations such as polymyxin B with doxycycline, polymyxin B with rifampicin, , tigecycline with polymyxin B and tigecycline-meropenem have been found to act synergistically *in vitro* against CRE(127,130).Colistin-gentamicin combination was

reported to be effective in a single study from a patientof endocarditis caused carbapenem resistant *Klebsiella pneumoniae*(132). Although *in vitro* synergy was not observed in fosfomycin-colistin combination, *in vivo* administration has found to lower mortality(133). In a recent study a combination of three drugstigecycline, colistin and meropenem was found to be associated with lower mortality in paitents with CRE infections (131).

Combination of drugs should ideally be tested *in vitro* for synergy before clinical use. Methods for *in vitro* synergy testing include the time kill assay, the checkerboard assay and the E-test methods. Controlled clinical trials are necessary to ascertain the optimal combination for therapeutic use(127).

Interestingly, it has been observed that carbapenems could still be effective in treatment of CRE especially in the strains with MICs in the lower range of resistance. CRE with carbapenem MICs $\leq$ 4μg/ml were still found to be susceptible to carbapenem both *in vitro* and *in vivo*. It is suggested that a high dose or prolonged infusion of a carbapenem preferably in combination with another active antimicrobial would ensure a favourable outcome. Amikacin, gentamicin and fosfomycin are a few antimicrobials which have shown synergy with carbapenems in CRE with low level resistance(134).

4.10 Clinical outcome

Infections with CRE tend to have adverse clinical outcomes like increased mortality, severity and duration of hospital stay.Numerous studies have been conducted

comparing the outcomes of CRE versus CSE infection in terms of mortality and in almost all studies mortality was found to be higher for CRE infection (Table 5).

Table 4.12:Comparative mortality rates of CRE versus CSE

Reference		**Chang *et al.*** (135)	**Ben david *et al*** (136)	**Mouloudi *et al.*** (137)	**Daikos *et al.*** (138)
Mortality Rate (%)	**CRE**	94%	48%	56 – 79* %	43%
	CSE	50%	17%	41%	16 – 19%

* MBLs were associated with lesser mortality than KPC

Various factors are known to influence the outcome of CRE infection independently. Microbial eradication and procedures done to remove the focus of infection improves recovery and survival. Borer *et al* in their study compared the outcome of CRE blood stream infections and CRE causing infections and other sites. They found that the mortality was higher in CRE bloodstream infections 72% than in other infections caused by CRE 22% (139).

Numerous other factors are also known to contribute to increased mortality in bloodstream infections such as advanced age and severity of the underlying disease. Astonishingly, Neuner *et al* inferred from their study that the time to initiate active treatment with antimicrobials showing *in vitro* susceptibility had no significant

influence on outcome of the disease. They also established that patients with CRE infections had a considerably longer duration of hospital stay. Also the recurrence rates of the disease is high, warranting readmission in patients who get discharged due to apparent initial improvement(140).

5. Materials and Methods

5.1 Study Design

This is an observational study conducted in the Department of Clinical microbiology, Christian Medical College and Hospital, Vellore. Samples received from patients for blood culture which were positive for screening test for carbapenem resistant *K. pneumoniae* and *E. coli* were included in the study.

5.2 Ethics approval

The approval for the study was obtained from the Institutional Review Board, CMC, Vellore.

5.3 Study duration

The study was conducted over a period of 18 months from January 2013 to June 2014.

5.4 Study samples

Blood culture was performed on the samples with the BacTAlert automated system (bioMe´rieux). Identification of Enterobacteriaceae from positive blood culture was done by cultural characteristics and biochemical methods.

Antimicrobial susceptibility by disk diffusion was performed as a part of the routine testing.

All isolates showing resistance showing resistance to imipenem /meropenem were included in the study to be evaluated for carbapenemase production.

5.4.1 Inclusion criteria

i. Any isolate identified as *E. coli* or *Klebsiella pneumoniae* from blood culture which was positive for initial screening tests for carbapenem resistance was included in the study.
ii. Multiple blood cultures received from a patient which grew the same isolate, only one of the isolates was included.
iii. For outbreaks, only one strain was included.

5.4.2 Exclusion criteria

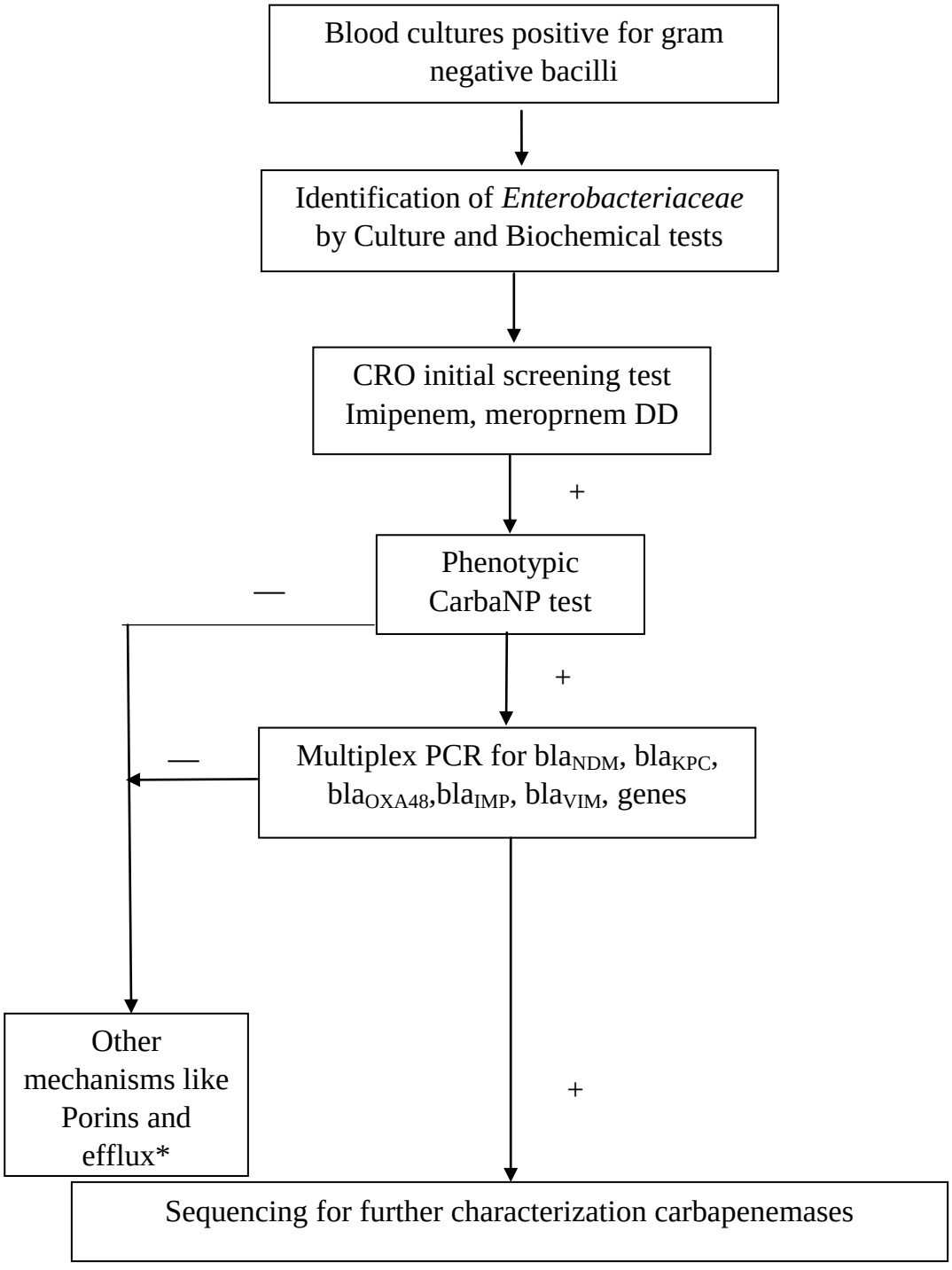
i. Isolates identified as *E. coli* or *Klebsiella pneumoniae* from blood culture which was negative for initial screening tests for carbapenem resistance was not included in the study.
ii. Isolates other than *E. coli* or *Klebsiella pneumoniae* were not included.
iii. Isolates identified as *E. coli* or *Klebsiella pneumoniae* from samples other than blood were not included.
iv. More than one isolate from the same patient was not included.

5.5 Data sources

Relevant demographic information about the patient, like age, sex, unit where the patient is admitted was obtained from clinical records. Testing of the study samples was done in the department of Clinical Microbiology.

5.6 Study algorithm

Diagrammatic representation of the study algorithm is shown below.



DD-disk diffusion

*not characterized in this study

5.7 Processing of study samples

The samples received for blood culture were loaded in theBacT/ALERT® automated Blood Culture system. When the bottle fagged positive for growth, a smear was made and stained with the Gram's stain and the broth was was subcultured onto Blood agar and MacConkey agar.

The gram negative bacilli were identified based on the cultural characteristics and preliminary screening media- mannitol motility medium, triple sugar iron agar, indole production and citrate utilization. Full characterization of the Klesiella spp and *E.* coli was done using biochemical tests.

Antimicrobial susceptibility testing was done by Kirby Bauer disk diffusion technique for first and second line antibiotics as per the standard operating protocol (Annexure)

The isolates found resistant to imipenem and meropenem were included in the study.

5.8 CarbaNP test

CarbaNP test was performed on all isolates included in the study by the following procedure:

A. Principle:

Based on a technique designed to identify the hydrolysis of the β--lactam ring of a carbapenem which is indicated by a colour change in the indicator phenol red.

B. Requirements

1. 1.5 ml Eppendorf tubes
2. Imipenem sodium salt (Sigma-Aldrich)
3. B-PERII, Bacterial Protein Extraction Reagent (Thermo Scientific, Pierce), Cat:78260.
4. ZnSO4, 7H2O (Sigma-Aldrich, Cat: 221376)
5. Negative (wild-type *E. coli*) and positive (*K. pneumoniae* OXA-48 or *K. pneumoniae* KPC-2) controls.

C. Preparation and storage of Solution A

1. A concentrated solution of red phenol 0.5% w/v was prepared.

2. 2 ml of the concentrated red phenol solution (vortexed strongly before pipetting to

resuspend the solution) was mixed in 16.6 ml of distilled water.

3. The pH was adjusted at 7.8 by adding drops of a NaOH solution (1 N)

4. 180 µl of ZnSO4 10 mM was added to this to obtain a finalconcentration of 0.1 mM.

Solution A is stable at room temperature for 1 week and may be kept at -20°C for several

months.

Solution A + imipenem (6 mg/ml) was prepared extemporaneously.

D. Procedure

1. 100 µl of 20 mM Tris-HCl lysis buffer (B-PERII, Bacterial Protein Extraction Reagent, Thermo Scientific, Pierce) was added in each of two 1.5 ml eppendorf tubes
2. 1/4 to 1/3 calibrated dose (10 µl) of bacterial colonies was resuspended in the 100 µl of 20 mM Tris-HCl lysis buffer. (Bacterial colonies may be recovered directlyfrom the antibiogram around disk of carbapenem performed according to the diskdiffusion techniques).
3. (i) 100 µl of Solution A in the first eppendorf tube and (ii) 100 µl Solution A + imipenem 6 mg/ml in the second 1.5 ml eppendorf tube were added.
4. The tubes were incubated at 37°C for a maximum of 2 hours
5. Optical reading of the colour of each tube was taken and interpreted as follows:

E. Interpretation

Interpretation:	**No antibiotic**	**Imipenem**
No carbapenemase	Red	Red
Carbapenemase producer	Red	Orange/Yellow
Not interpretable	Yellow	Yellow

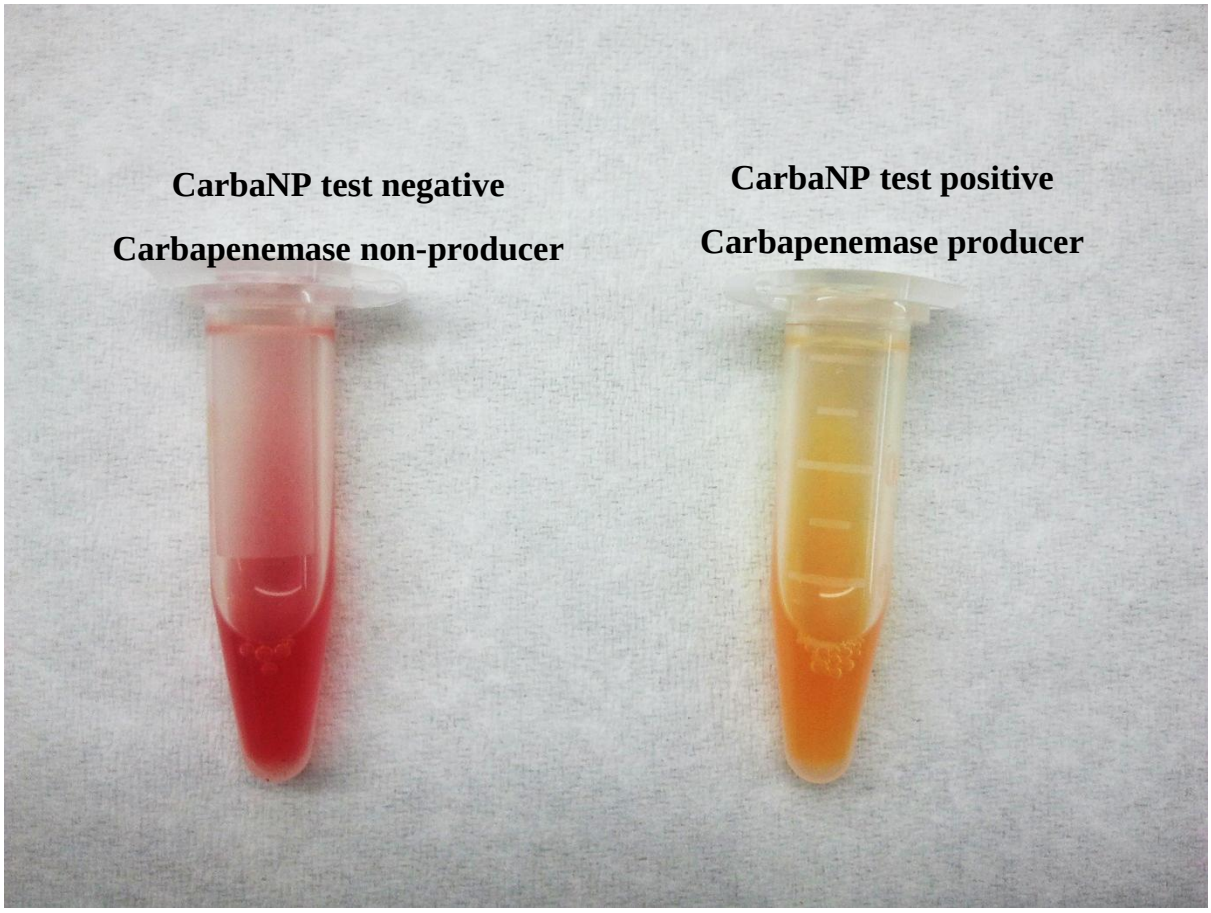


Fig 5.1: Carba NP test

Usually, the time required for obtaining positive results was as follows:

1. KPC producers: 2 to 30 min
2. OXA-48 like producers: 20 min to 1h
3. Metallo-β--lactamases (NDM, VIM, IMP): 15 min to 1h

5.9 Multiplex PCR

5.9.1 DNA extraction from the isolates

5.9.1.1 . Materials required

Suspenstion of isolates in nuclease free PCR grade water (200 µl)

QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) containing:

Proteinase K

Lysis buffer AL

Wash buffer 1 AW1

Wash buffer 2 AW2

Elution buffer AE

QIAamp Mini Spin Columns

Collection Tubes (2 ml)

Ethanol

Nuclease free water

Dry bath (temperature to be set at 56°C)

B. Procedure

DNA extraction was performed in the 'PCR dirty' room as per manufacturer's instructions.

1. Qiagen Proteinase K (20µl) was taken in the bottom of a 1.5 ml microcentrifuge tube.

2. To it 200µl serum sample was added to the microcentrifuge tube.

3. Then, 200µl Buffer AL (lysis buffer) was added to the sample in the same tube.

4. These substances were mixed well by pulse vortexing for 15 seconds.

5. The tube was incubated at 560C for 10 minutes.

6. After this, the tube was briefly centrifuged to remove the drops from inside the lid.

7. To the tube, 200µl of absolute alcohol was added to the tube.

8. Again, it was mixed well by pulse vortexing for 15 seconds.

9. Then, the tube was briefly centrifuged to remove the drops from inside the lid.

10. The mixture was placed in the QIAamp spin column with a 2 ml collection tube without wetting the rim and the cap was closed.

11. The spin column was centrifuged at 8000 rpm for 1 minute.

12. The QIAamp spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.

13. The QIAamp spin column was carefully opened and 500µl Buffer AW1 (Wash buffer) was added to it without wetting the rim and the cap was closed.

14. It was centrifuged at 8000 rpm for 1 minute

15. The QIAamp spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.

16. The QIAamp spin column was carefully opened and 500µl Buffer AW2 (Wash buffer) was added without wetting the rim and the cap was closed.

17. It was centrifuged at full speed of 14,000 rpm for 3 minutes.

18. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded.

19. The QIAamp spin column was carefully opened and 200µl Buffer AE (eluting buffer) was added to it.

20. The tube was incubated at room temperature for 1 minute.

21. It was then centrifuged at 8000 rpm for 1 minute and the spin column was discarded.

22. The 1.5 ml centrifuge tube contained DNA which was labelled and stored at -700C in two aliquots.

5.9.2 Detection of genes by multiplex PCR

Principle:

Polymerase chain reaction amplifies a specific target region of the template DNA strand. This produces DNA fragments of precise lengths giving discreet electrophoretic patterns based upon their sizes. Using suitable primers and cycling conditions the five gene targets were amplified ina single reaction. The amplicons were visualized in 2%agarose gel and were detected depending upon their base pair sizes on electrophoresis.

PCR

A. Primers

The lyophilised primers used for the multiplex PCR were reconstituted in Tris EDTA (TE) buffer and stored in aliquots at -70°C. The primer sequences were as follows:

Table 5.1. Primer sequences used for multiplex PCR

Primer name	**Primer sequence(5ˈ-3ˈ)**	**PCR product size(bp)**
IMP-F	GGAATAGAGTGGCTTAAYTCTC	189
IMP-R	CCAAACYACTASGTTATCT	
VIM-F	GATGGTGTTTGGTCGCATA	390
VIM-R	CGAATGCGCAGCACCAG	
OXA-48F	TATATTGCATTAAGCAAGGG	800
OXA-48R	CACACAAATACGCGCTAACC	
KPC-F	TGTCACTGTATCGCCGTC	1011
KPC-R	CGGGTTGGACTCAAGACG	
NDM-F	GGTTTGGCGATCTGGTTTTC	984
NDM-R	CGGAATGGCTCATCACGATC	

B. **Preparation of primer mix**

A primer mix was prepared to dilute the 100µM concentration of the primers to the optimal concentration to be used in the multiplex PCR reaction. The primer mix was prepared in sterile 1X Tris EDTA buffer as below:

Components	Required amount (µl)	Final primer concentration
IMP-F (100 µM)	2 µl	2 µM
IMP-R (100 µM)	2 µl	2 µM
VIM-F (100 µM)	2 µl	2 µM
VIM-R (100 µM)	2 µl	2 µM
NDM-F (100 µM)	2 µl	2 µM
NDM-R (100 µM)	2 µl	2 µM
OXA-48 F (100 µM)	2 µl	2 µM
OXA-48 R (100 µM)	2 µl	2 µM
KPC-F (100 µM)	2 µl	2 µM
KPC-R (100 µM)	2 µl	2 µM
TE buffer	80 µl	
Total volume of mix	100 µl	

C. Master mix preparation

The concentrations of the different components of the PCR mix for one reaction are given below:

Components	Required amount (µl)	Final concentration
2x multiplex master mix (Qiagen)	10 µl	1x
5x Q solution	2 µl	0.5x
10x Primer mix (containing each primer at a concentration of 2 µM)	2 µl	0.2 µM final concentration
DNA template	1 µl	Not to exceed 0.4 µg
dH2O	to 20 µl	
Total volume of reaction	20 µl	

D. Procedure for amplification

1. The master mix was prepared for the appropriate number of reactions with the above template. The master mix was prepared in the clean room or 'DNA-free' room.

2. Appropriate number of 0.5ml PCR tubes were labeled appropriately, and the master mix was distributed in them.

3. DNA extracts were removed from the storage area, brought to room temperature and spun briefly in a microcentrifuge.

4. 1 µl of DNA was added and the final volume was 20 µl. Addition of DNA was done in the 'dirty room'.

5. Nuclease free water was used as negative control after every two samples.

6. Amplification reactions were carried out in Veriti ™ Thermal Cycler (Applied Biosystem, Foster City, California, USA)

7. The cycling conditions were:

Initial denaturation:	95° C	15 min	- 1 cycle
Denaturation:	94° C	30 sec	- 30 cycles
Annealing:	59° C	1.5 min	
Extension:	72° C	1.5 min	
Final extension:	72° C	10 min	– 1 cycle

E**. Post amplification DNA detection by Gel Electrophoresis:**

1. Two microliters of each amplicon was mixed with 1 µL of 6X loading dye bromophenol blue.
2. The amplified products were then subjected to electrophoresis in freshly prepared 2% agarose gel containing 0.5 µg/ml ethidium bromide.
3. Test samples and negative control were loaded in appropriate wells. Molecular ladder (DNA Marker-A, Bio Basic Inc.Canada) used was 100-1000 bp long.
4. The electrophoresis was done at 100 volts for 75 minutes.
5. The gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, Hercules, California, USA).
6. The positive and negative controls of each run were checked and the run was validated. Then the results of the samples were taken

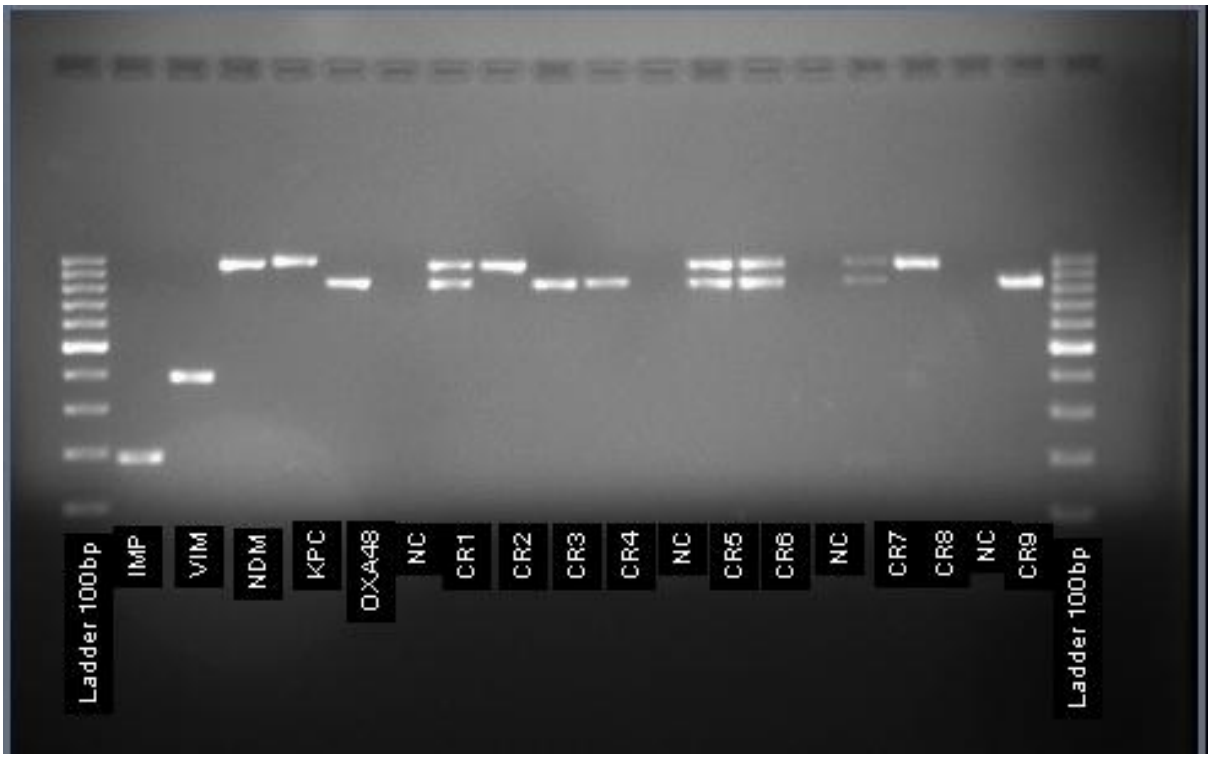


Figure 5.2: Documentation under UV light following amplification

Lane 1 and 20: 100 to 1000 bp molecular ruler

Lanes 2-6: Positive controls for bla_{IMP}189bp, bla_{VIM}390bp, bla_{NDM} 984bp, bla_{KPC}1011bp, $bla_{OXA48\ like}$800bp bands.

Lane 8, 13, 14 and 16: Sample positive forbla_{NDM} and $bla_{OXA48\ like}$ genes

Lane 10, 11, 17: Samples positive bla_{NDM}genes

Lane 10, 11, 19: Samples positive for $bla_{OXA48\ like}$ genes

Lanes 7, 12, 15, 18: Negative controls

5.10 Identification of enzyme variants polymerase chain reaction-sequencing

A representative set of samples (20 samples positive for *bla*$_{\text{OXA48}}$ and 7 samples positive for *bla*$_{\text{NDM}}$ gene) were bi-directionally sequenced by Sanger sequencing for verification of PCR results and to determine the enzyme variants.

Principle:

This is a 'chain termination' method of DNA sequencing to determine the precise order of nucleotides in a segment of DNA. After amplification of the target region, the products are purified in order to remove extra dNTPs and primers (Pre-cycle sequencing clean-up). This is followed by PCR to synthesize single stranded DNA templates. The reaction mix for the PCR contains, along with the four deoxynucleotide triphosphate (dNTPs), limited amounts of different dideoxy nucleoside triphosphate (ddNTPs). These ddNTPs get incorporated into the chain by DNA polymerase, as efficiently as dNTPs. However, as they lack the 3'-OH group required for attachment of the next nucleotide, their incorporation brings about chain termination. This process generates fragments randomly that differ in length by one base pair. In automated cycle sequencing using dye terminator chemistry, the ddNTPs are tagged with different fluorescent dyes, each emitting light at a unique wavelength when excited by a laser. As the products are subjected to capillary electrophoresis in a genetic analyzer, a laser excites the fluorescent dye labels at the 3' ends of the ddNTPs, and depending upon the unique wavelength emitted, the nucleotide is identified as adenine (A), cytosine (C), guanine (G) or thymine (T). The emitted fluorescence is recorded by a camera and a software converts the data to a colour coded electropherogram, in which blue represents C, green represents A, *bla*ck represents G and red represents T.

A. DNA amplification by PCR

1. Each gene-specific primer mix was prepared in TE buffer and contained 5 □ M of the forward primer and reverse primer.The primers used for sequencing were as follows

Gene	Name of primer	Primer sequence 5'-3'	Product size(bp)
NDM	preNDMf	CACCTCATGTTTGAATTCGCC	984 bp
	preNDMr	CTCTGTCACATCGAAATCGC	
OXA-48like	preOXA-48f	TATATTGCATTAAGCAAGGG	800 bp
	preOXA-48r	CACACAAATACGCGCTAACC	

2. The master mix preparation was done in the same manner as PCR reaction above. The cycling conditions were also the same.

3. Amplification of produced a 984bp for base pairs for *bla*$_{NDM}$ and 800bp for *bla*$_{OXA48\ like}$ gene.

4. Electrophoresis of the amplified products was done on 2% agarose gel.

5. The gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, Hercules, California, USA).

B. Pre-cycle sequencing clean up

It was done according to the protocol given by HighPrep PCR (Magbio Genomics, Inc. Canada)

1. The HighPrep PCR reagent was brought to room temperature for at least 30 min before use. It was shaken thoroughly to fully resuspend the magnetic beads.

2. To 20µl of PCR amplicon, 36µl of HighPrep PCR reagent was added and mixed thoroughly with a pipette 6-8 times in a 1.5 ml eppendorf tube.

3. It was incubated at room temperature for 5 minutes.

4. The sample tubes were placed on a magnetic separation device for 3 minutes until the solution clears.

5. With the samples still on the magnetic separation device, the supernatant was removed and discarded with a pipette.

6. Then, 200µl of 70% ethanol was added to each sample tube without removing the tubes from the magnetic separation device.

7. The tube was incubated on the magnetic separation device for 30 seconds at room temperature.

8. The clear supernatant was removed and discarded with a pipette.

9. Again, 200µl of 70% ethanol was added to each sample tube without removing the tubes from the magnetic separation device.

10. It was incubated on the magnetic separation device for 30 seconds at room temperature.

11. The clear supernatant was removed and discarded with a pipette.

12. The beads were dried by incubating at room temperature for 5 minutes with the plate still on the magnetic separation device.

13. The samples were removed from the magnetic separation device.

14. Finally, 40 µl of water was added to each tube and it was mixed thoroughly five times with a pipette.

15. The samples were placed back on the magnetic separation device and incubated at room temperature for 1 minute.

16. The elute was taken in new 0.5 µl tubes and used for PCR for sequencing and gel documentation to detect the product.

C. PCR for Sequencing

1. Sequencing reactions were carried out for the forward and reverse strands using ABI Prism BigDye® terminator v3.1 cycle sequencing reagents.

2. The reaction volumes were as follows

Reagent	Volume per reaction (µl)
Ready reaction (RR) mix	1
5 mM MgCl2	3
Primer (Forward/Reverse)	2 (5µmol/µl)
Purified PCR product	2
Nuclease free water	2
Total volume	10

3. The cycling conditions were

96°C for 15 seconds
50°C for 20 seconds
60°C for 4 minutes
} 25 cycles

D. Post cycle sequencing clean-up

1. 10µl of DTR reagent was taken in a 1.5ml eppendorf tube.

2. To it 10µl of amplified PCR product and 40µl of 85% ethanol were added and mixed well ten times with a pipette.

3. The tube was incubated for 5 minutes at room temperature on the magnetic separation device.

4. The supernatant was discarded with a pipette.

5. Then, 100µl of 85% ethanol was added to each tube keeping it on the magnetic separation device without mixing.

6. It was incubated for 2 minutes at room temperature on the magnetic separation device and the supernatant was discarded.

7. Again, 100µl of 85% ethanol was added to each tube keeping it on the magnetic separation device without mixing.

8. It was incubated for 2 minutes at room temperature on the magnetic separation device and the supernatant was discarded.

9. The cap was opened and the beads are allowed to dry by incubating at room temperature for 10 minutes.

10. Finally, 40µl of injection solution was added to it and mixed well.

11. The tubes were incubated at room temperature for 5 minutes on the magnetic separation device.

12. The clear supernatant was taken and loaded in the sequencing plate.

E. Sequencing

The sequencing plate was loaded in the sequencer to obtain the genomic sequences. The ABI 310 Genetic analyser (Applied Biosystems, Foster City, CA, USA) was used to enumerate the sequences.

F. Analysis

The sequence obtained was visualised and edited using Finch TV (Perkin Elmer, Seattle, WA) to attain the final sequence for analysis.

The homology of the sequence obtained was compared with that of the existing $bla_{\text{OXA48 like}}$ and bla_{NDM} genesequence in the Gene Bank . This was performed using the basic local alignment search tool (*BLA*ST, available from www.ncbi.nlm.nih.gov/*BLA*ST) programme with the available standard reference sequences in the Gene Bank for homology.

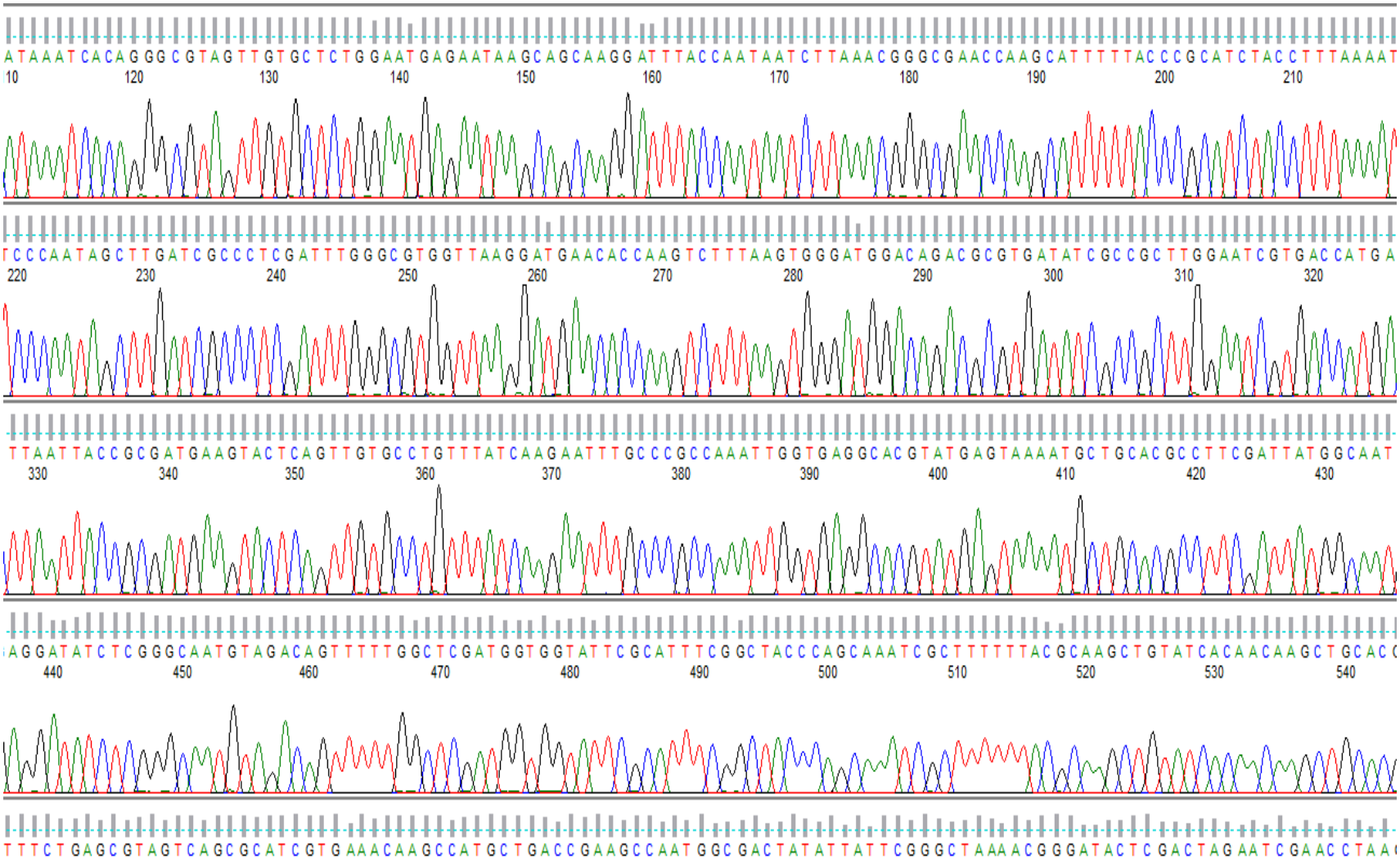


Figure 5.3: Sequence analyzed in FinchTV - DNA sequence chromatogram trace viewer for *bla*$_{\text{OXA48 like}}$ gene.

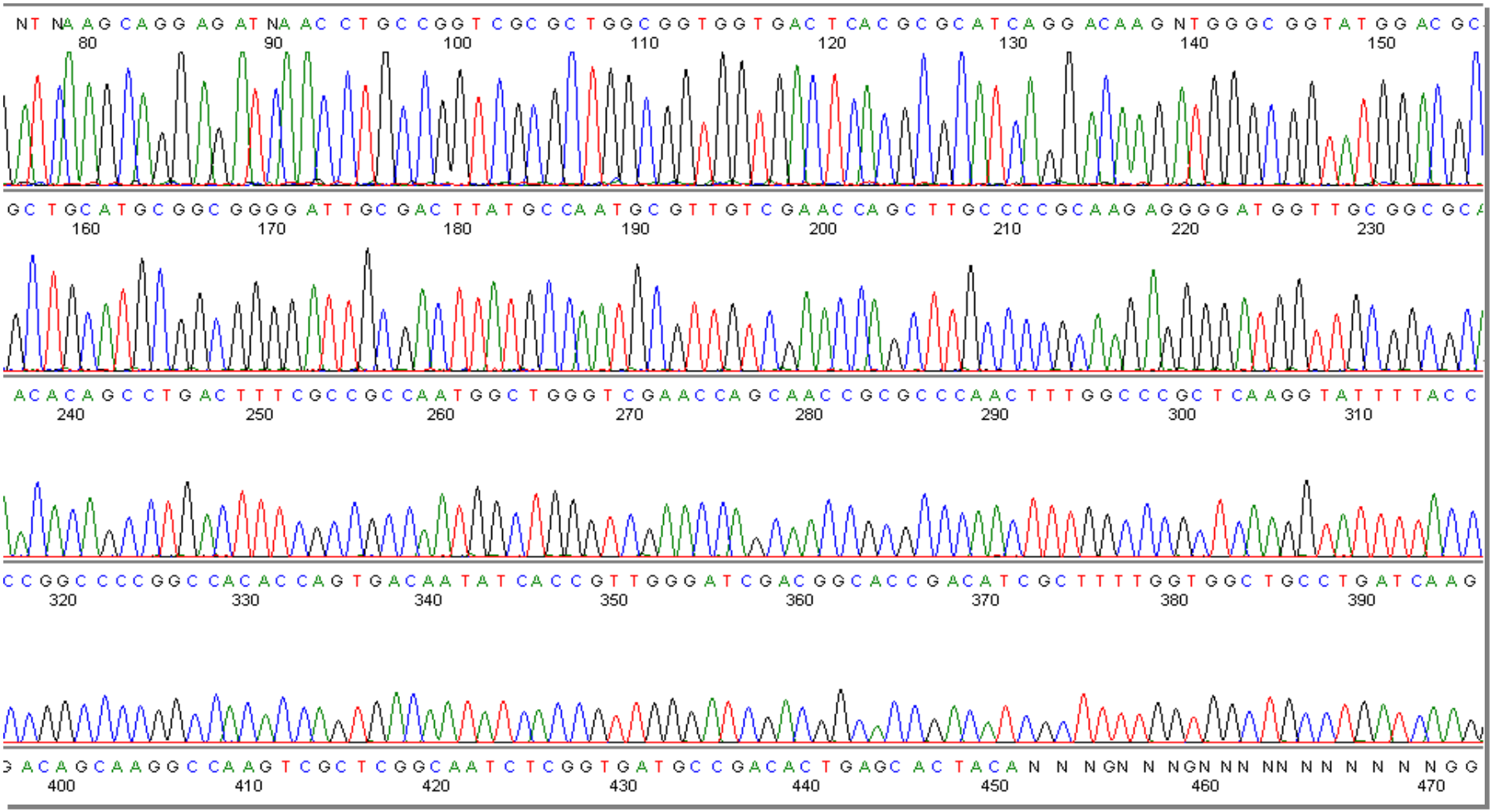


Figure 5.4: Sequence analyzed in FinchTV - DNA sequence chromatogram trace viewer for *bla*$_{NDM}$ gene.

6. Results

6.1 Demographic data

During the period of the study from January 2013 to December 2013 over 41,168 samples were received for blood culture.

11.1% (n=4585) samples received for blood culture were positive with growth of a pathogen.

44% (n=2031) of the isolates were gram positive cocci 50% (n=2266) were gram negative bacilli, 6% (n=270) were *Candida spp*. And 0.3% (n=18) were *Cryptococcus spp.* (fig6.1)

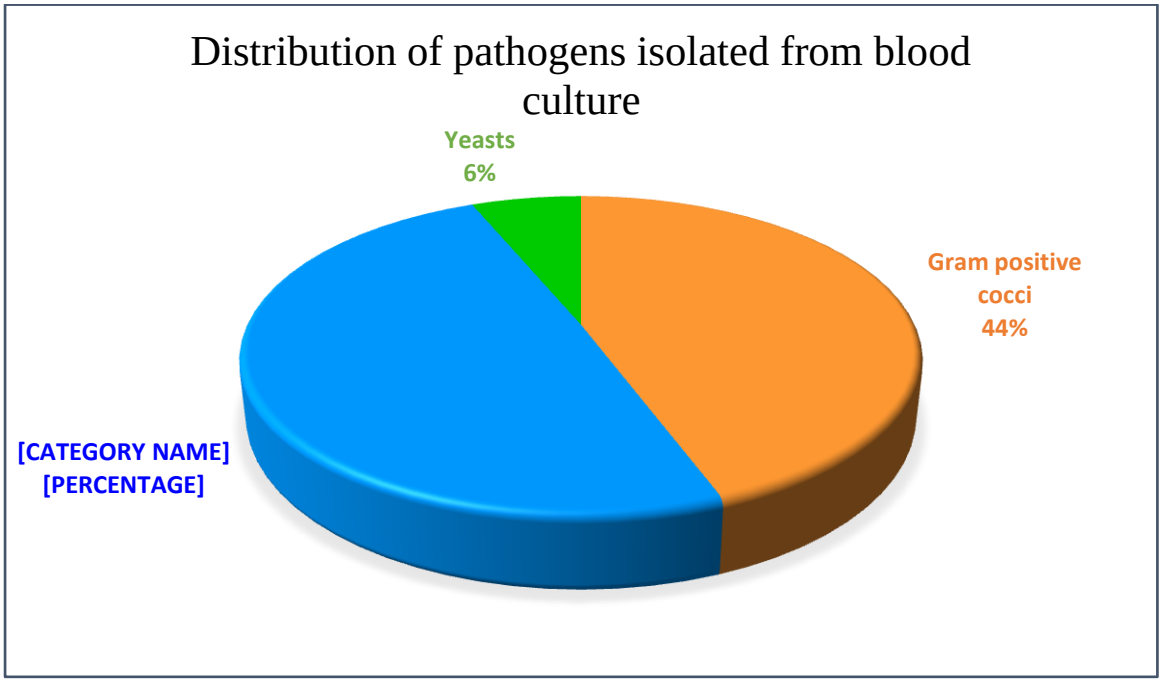


Fig 6.1Distribution of pathogens isolated from blood culture

The gram negative bacilli included *Klebsiella spp, E. coli, Enterobacter spp, Proteus spp., Salmonella Typhi, Salmonelalla Paratyphi A, Non- typhoidal Salmonella, Brucella spp, Pseudomonas aeruginosa, Burkholderia pseudomallei and other Non fermenting gram negative bacilli* (NFGNB). The percentage distribution of these isolates are shown in figure 6.2

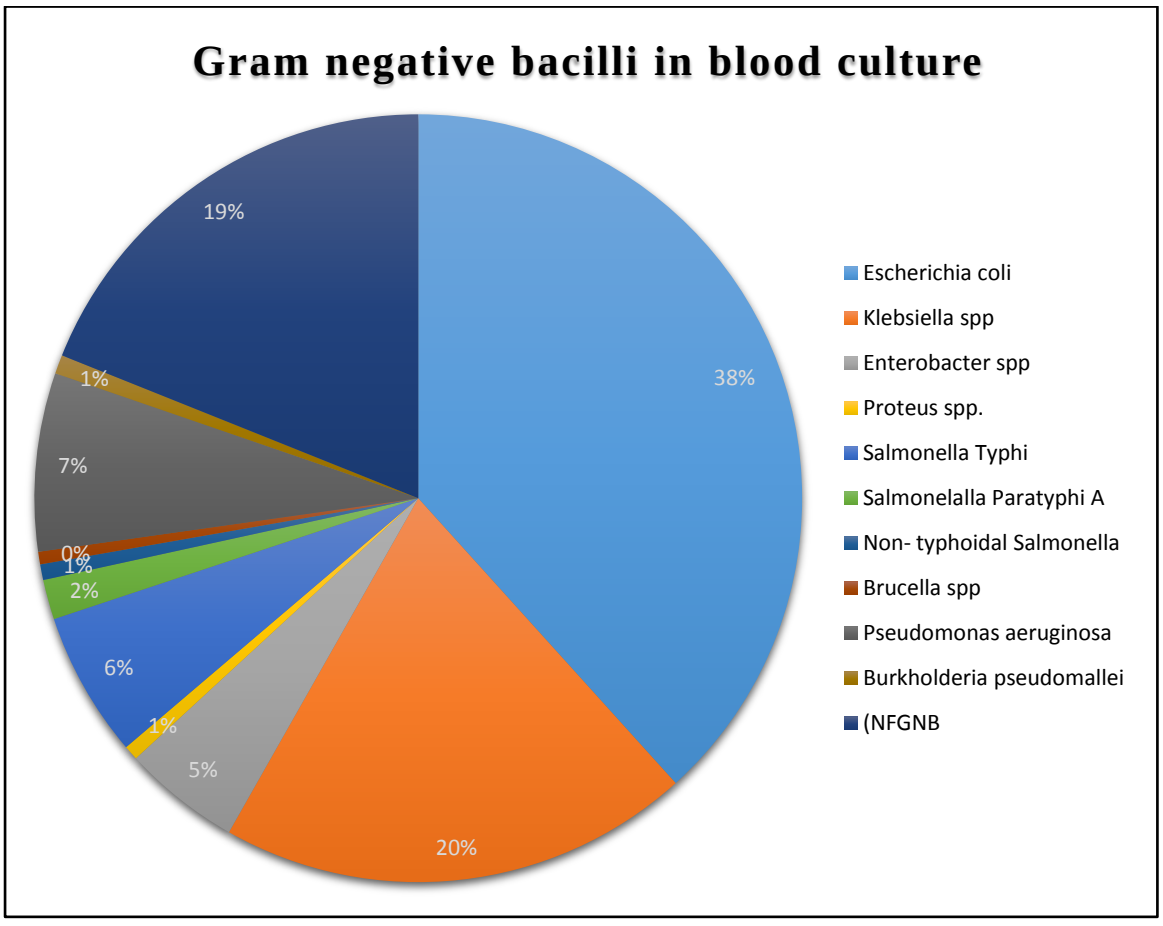


Fig. 6.2 Gram negative bacilli isolated from blood culture

A total of 451*Klebsiella pneumoniae* and 868*E. coli* were isolated from January 2013 to December 2013 which was the duration of the study.

20% (n=90) of the *Klebsiella pneumoniae* isolates and 3.6% (n= 32) were positive in the screening test for carbapenem resistance.

After considering the inclusion and exclusion criteria as mentioned in materials and methods section, 122 isolates were included in the study.

All the samples included in the study belonged to the family *Enterobacteriaceae* and were resistant to imipenem, meropenem on screening with disk diffusion technique.

116 samples were received from hospitalized patients and 6 were received from casualty.

58% (n=71) of the inpatient samples were from the wards and 37% (n=45) were from the intensive care units and high dependency units. (fig 6.3)

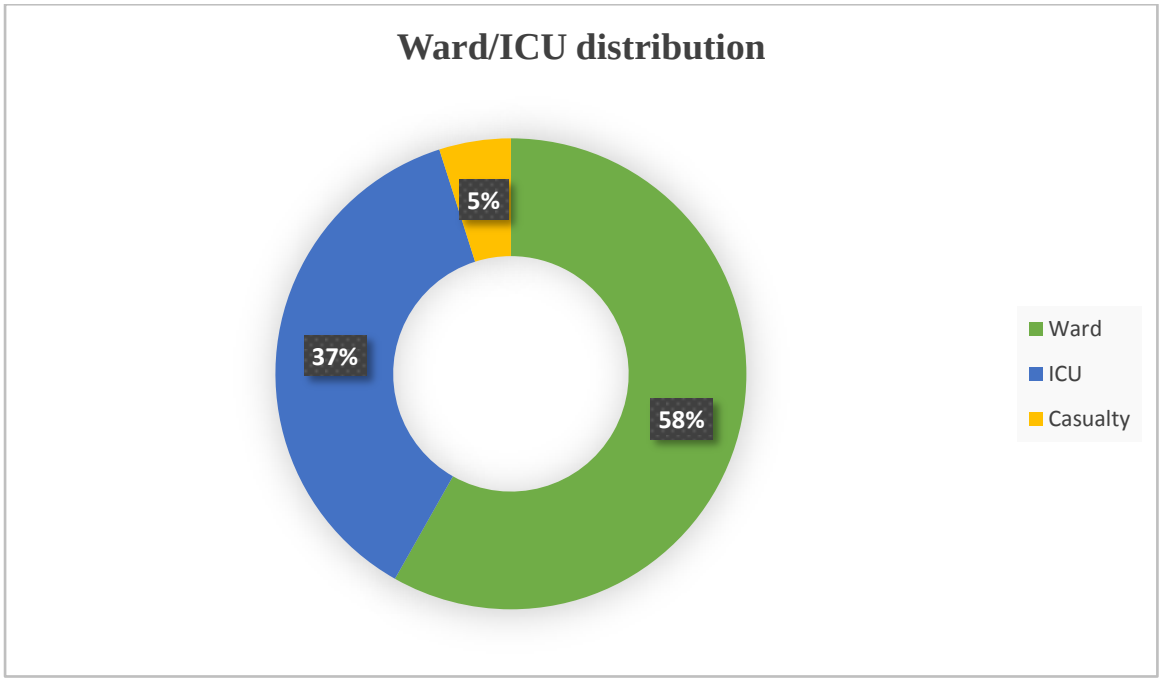


Fig.6.3 Ward and ICU distribution of patients

Majority of the samples were from the hematology unit 33% followed by 23% from the various surgical units such as general surgery, hepatobiliary surgery, gastrointestinal surgery. 9% of the samples were from the paediatric units and 5% were from the oncology units. The remaining samples were from nephrology, reproductive medicine, nephrology, neurology, thoracic surgery, orthopaedic surgery, cardiovascular surgery and physical medicine and rehabilitation. (Fig.6.4)

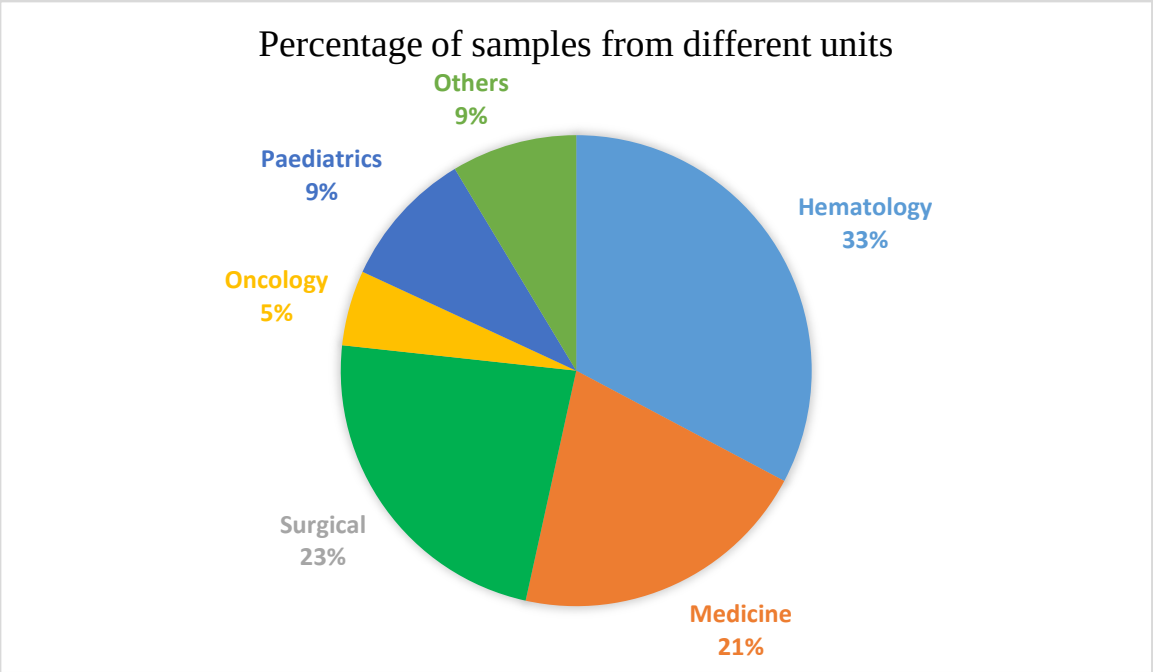


Fig.6.4 Percentage of samples from different units

75 isolates (61%) were from males and 47 (39%) were from females (Fig.6.5). The sex distribution was found to be slightly on the higher side for males. The age of the patients ranged from one day old newborns to 84 years. When observed for age distribution, maximum number of isolates was from adults between 20 and 60 years (Fig.6.6)

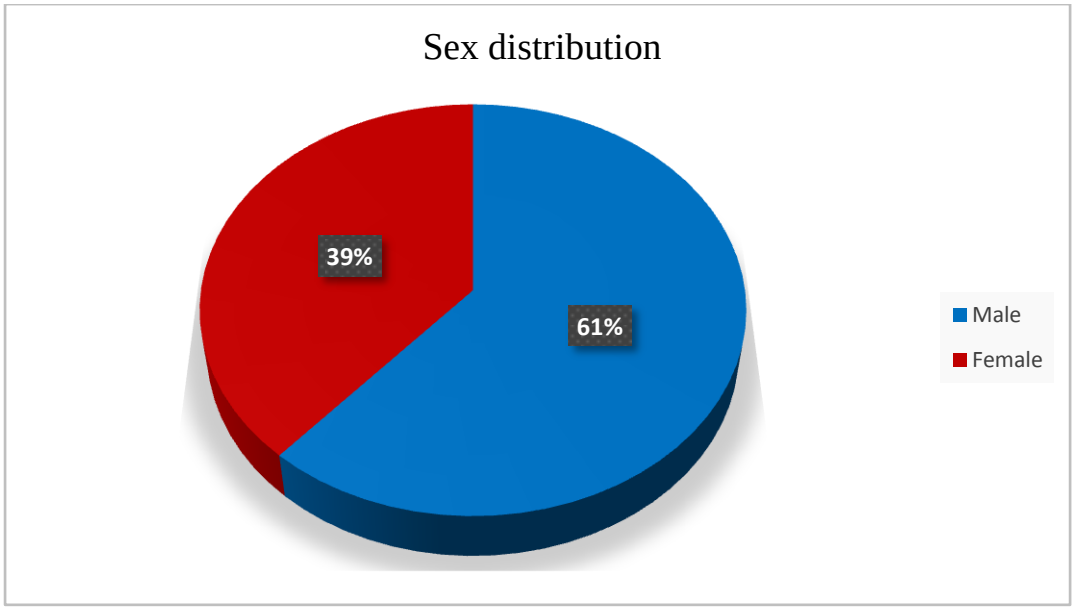


Fig.6.5: Sex distribution of patients

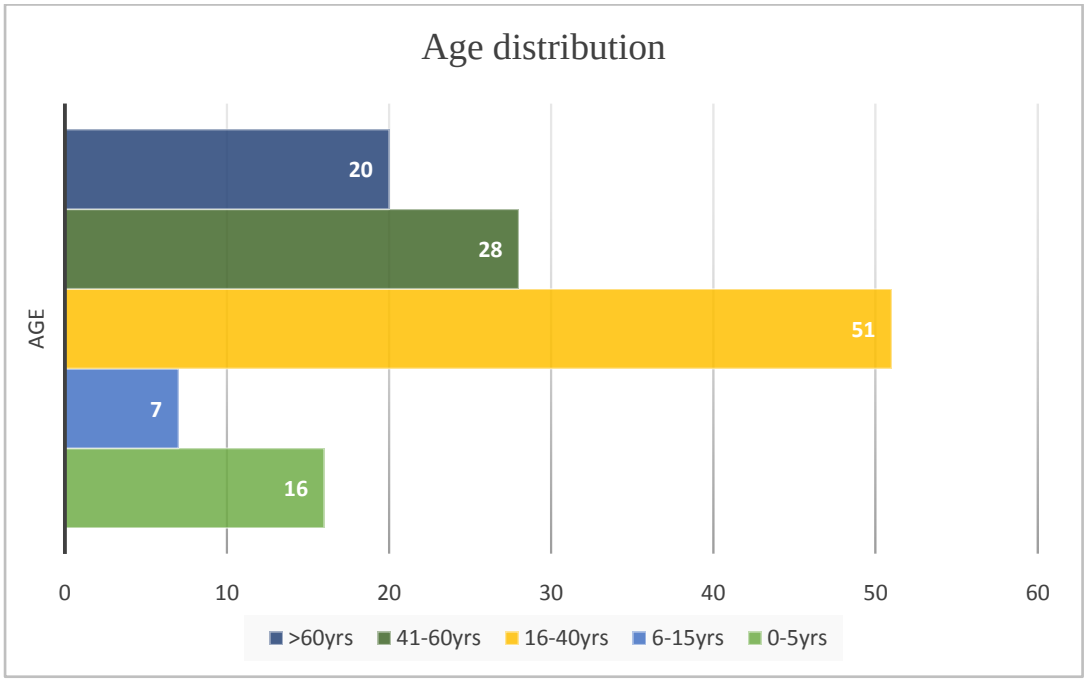


Fig.6.6 Age distribution of patients

6.2 Microbiological data

6.2.1 Identification of the pathogens

Of the 122 isolates 74% (n=90) were identified as *Klebsiella pneumoniae* and 26% (n=32) were *E. coli.*

6.2.2 Antimicrobial susceptibility profile

All the isolates were tested for resistance to carbapenems using imipenem or meropenem disk diffusion technique and the zone sizes were interpreted based on CLSI 2013 guidelines. The antimicrobial susceptibility profile of the isolates was determined for the first line antimicrobial agents by the disk diffusion technique (Fig.6.5). All the 122 (100%) isolates were resistant to impenem and meropenem by disk diffusion testing. All the isolates were also resistant to other β- lactam antibiotic cefpodoxime and β- lactam/β- lactamase inhibitor combination piperacillin-tazobactum, cefoperazone-sulbactum. 6%(n=7) isolates were susceptible to gentamicin, 10% (n=12) isolates were susceptible to amikacin and netilmicin. 4% (n=5) isolates showed susceptibility to ciprofloxacin. 99% (n=121) of the isolates were susceptible to colistin. (Fig. 6.7)

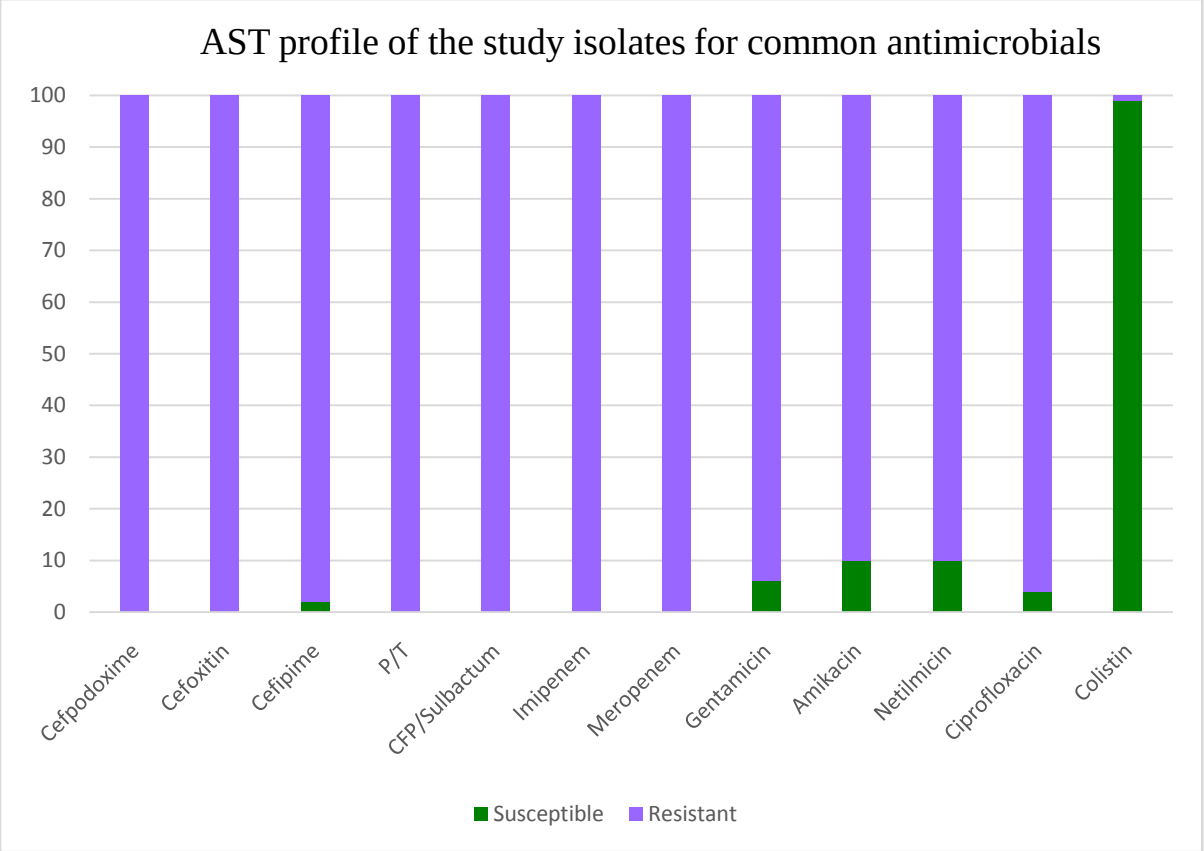


Fig. 6.7AST profile of the study isolates for common antimicrobials

6.2.3 CarbaNP test

All isolates were subjected to CarbaNP test to phenotypically differentiate the carbapenemase producers and carbapenemase non-producers. The change of colour of phenol red indicator to yellow/orange was taken as positive for carbapenemase production. Carbapenemase production was noted in 93% (113 out of 122) of the isolates with this test. The test was repeated on the negative isolates with an increased incubation of the bacterial isolate in the lysis buffer. On this modification four of the negative isolates tested positive thus a total of 96% (117 out of 122) isolates tested positive. Five isolates remained negative even on repeat testing.

The five isolates which were negative by CarbaNP test, three isolates were *Klebsiella pneumoniae* and two were *E. coli.* Among the five negatives, onewas positive for $bla_{\text{OXA48 like}}$ gene and two were positive for bla_{NDM} gene by PCR. Of the CarbaNP negative isolates one was also negative by PCR and which was an *E. coli* isolate.

6.2.4 Multiplex PCR for bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC}, $bla_{\text{OXA48 like}}$ genes, sequencing and BLAST matching.

A multiplex PCR was done on all the study isolates to detect the presence of bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC}, $bla_{\text{OXA48 like}}$ genes. An isolate was considered positive for the gene when its amplified products produced the specific band after electrophoresis. The position of the band was verified with the 100- 1000bp molecular ladder which was run with every set of amplicons. Isolates which did not possess the gene did not produce any band. 112 isolates (92%) were found to possess at least one of the genes.

40% (n=49) of the isolates were found to possess the bla_{NDM} gene, 39% (n=48) isolates were positive for $bla_{\text{OXA48 like}}$ gene. 12% (n=15) of the isolates showed the coexistence of both $bla_{\text{OXA48 like}}$ and bla_{NDM} genes. None of the isolates were positive for bla_{IMP}, bla_{VIM} genes. 8%(n=10) isolates did not show the presence of any of the genes. (Table.6.1)

20 isolates which were positive for the *bla*OXA48gene and 7 isolates positive for bla_{NDM} gene were randomly selected, sequenced, BLAST matched with the reference sequences in GenBank. All the 20 (100%) of the $bla_{\text{OXA48 like}}$ genes were found to be bla_{OXA181} a variant of the $bla_{\text{OXA48 like}}$gene. The 7 bla_{NDM} gene positive were found to

be $bla_{\text{NDM}\ 1}$ variant. The sequences showed 100% identity with the reference sequences.

Table.6.1 Molecular characterization of the isolates by PCR

Gene	No.of isolates (n=122)	Percentage
bla_{NDM}	49	41
$bla_{\text{OXA48 like}}$	48	39
bla_{KPC}	0	0
bla_{NDM} $_{+}bla_{\text{OXA48}}$ like	15	12
bla_{VIM}	0	0
bla_{IMP}	0	0
Negative	10	8

6.3 Characterization of resistance mechanisms:

Among the 122 isolates positive for phenotypic screening test for carbapenem resistance by disk diffusion for imipenem and meropenem, 117 were found to be

carbapenemase producers by CarbaNP test and 112 were found to possess at least one of the five carbapenemase genes. (Table 6.2)

Disk diffusion test	CarbaNP test	PCR	No. of isolates
+	+	+	112
+	-	+	4
+	+	-	9
+	-	-	1

Table. 6.2 Characterization of carbapenem resistance of the study isolates

7. Discussion

7.1 Carbapenem resistant Enterobacteriaceae

In this study the organisms *Klebsiella pneumoniae* and *E. coli* were chosen to represent the family *Enterobacteriaceae* as they are the most commonly associated with acquisition and spread of plasmid mediated carbapenemase genes. Blood stream infection is an invasive infection and requires prompt identification of organisms and accurate therapy. The *K. pneumoniae* and *E. coli* isolates causing blood stream infection would truly represent the burden of invasive disease caused by these organisms and impact of resistance mechanisms on the outcome of the disease.

As only *K. pneumoniae* and *E. coli* isolates were included in the study the overall rate of carbapenem resistance among *Enterobacteriaceae* considering the other genera belonging to this family was about 15% (data not shown/published). This data was only derived from the bloodstream isolates. This was at par with those reported from other centres in Worldwide and in India. SMART study conducted in 2009, where intra-abdominal isolates were screened for carbapenem resistance showed the worldwide prevalence of 28% (10). A multicentric study conducted in various Indian cities in 2006-2007 where *Enterobacteriaceae* isolates were collected from 14 hospitals, showed an overall prevalence of only 2.7%(113). The studies published more recently suggest that the burden of CRE has steadily risen since then. Dutta *et al.* analysed ten year resistance profile of their blood stream infections and observed that the carbapenem resistance in *K. pneumoniae* had risen from 2.4% in the year 2001 to an alarming 52% in 2009(121). The differences in the incidence of CRE are due to

differences in sites from where the samples have been collected, the usage of carbapenem antibiotics for treatment and existence of hospital infection control practices. Our hospital is equipped with an active hospital infection control committee to prevent nosocomial infections. The reason for high prevalence of carbapenem resistance in a tertiary care center could also be attributed to the fact that patients are referred from different hospitals after failure of therapy with multiple antibiotics. The possibility of a community acquired CRE infection cannot be ruled out in our country as carbapenem resistant *Enterobacteriaceae* have been found contaminating water sources (141).

Blood stream infection due to *E. coli* was more common than *K. pneumoniae* but carbapenem resistance was higher in the *K. pneumoniae* isolates. This was also observed in various other studies. In a study by Baroud *et al.* where the resistance to carbapenems was higher in *K. pneumoniae* 2.45% when compared with *E. coli* 1.07% (142).

Majority of the isolates (95%) were obtained from in-patients who have been hospitalized either in the wards or intensive care units with only a minor contribution from out-patients, which correlates well with the fact that these organisms are nosocomial pathogens (10). Similar results have been seen in studies worldwide. Most of these organisms in the studies have been isolated from critically ill hospitalized patients who have higher exposure to prior antimicrobial therapy and association with indwelling devices (136,137).

Highest proportion of the in-patients were from the Hematology unit. The risk of developing nosocomial blood stream infections in patients with underlying

haematological malignancies is higher, especially in patients who have undergone transplantation. The incidence rate was found to be 11-38% in the study by Wissplinghoff *et al* (143).

In this study the screening test used for identification of carbapenem resistant *Enterobacteriaceae* was disk diffusion testing for imipenem and meropenem. The results of the susceptibility testing was interpreted using CLSI 2013 guidelines. CLSI revised its carbapenem disk diffusion and MIC breakpoints for *Enterobacteriaceae* in 2010, following which it was recommended that with the use of new breakpoints modified hodge test for confirmation of carbapenemase production was necessary to be done only for hospital infection control and epidemiological purposes.

7.2 Antimicrobial susceptibility profile

The study isolates were tested for the routinely used first line antimicrobial agents and found to be resistant to most of them. This finding of resistance to first line drugs has been observed in most of the studies done on CRE around the world. Among the first line agents, a small proportion of the isolates were susceptible to gentamicin, amikacin, netlmycin and ciprofloxacin. The susceptibility of CRE to aminoglycosides has been reported in many studies. This high susceptibility rate of CRE to aminoglycosides is infrequent in Indian studies whereas has been noted in studies from the USA and European countries (118)(144)(145). This could due to the fact that, the enzyme endemic in Indian subcontinent is NDM. Organisms bearing variants of NDM have been found to be inherently resistant to aminoglycosides. On the other

hand it is a relatively infrequent feature of the strains producing KPC and other MBLs which are more prevalent in the USA and European countries (112)(144)(140).

Most of the isolates were susceptible to colistin. This finding correlates with many studies conducted on CRE around the globe (146)(10). Even though the isolates remain susceptible colistin resistanceorganisms have been observed. This drug has to be used with caution because colistin monotherapy is associated with selection of resistant mutants (127).

7.3 Detection of the different mechanisms of carbapenem resistance

When tested with the CarbaNP test, 93% of the test isolates tested positive for the production of carbapenemase. The sensitivity of this test has been widely evaluated and found variable. Dortet *et al.* and Huang *et al.* in their study found the sensitivity of the CarbaNP test to be 97.9% and 97% respectively (66,67) . On the other hand Tiget *et al.* found the sensitivity to be 76% which was much lower than described earlier. The sensitivity was observed to increase to 80% when concentrated bacterial extract was used (63). A similar phenomenon was observed in our study when a concentrated bacterial extract was used with prolonged incubation in the lysis buffer, 96% of the isolates gave a positive result. As the strains which initially gave a negative test were mucoid strains, false positive reactions with mucoid strains used in this test are well documented (147).

Tiget *et al* also concluded that the CarbaNP test was less sensitive for isolates carrying OXA- 48 like enzyme and reported a sensitivity of 55.7% for OXA-48 like producers

using the original protocol and 70.9 % using the modified protocol. There are several other studies which have established good sensitivity of the test with OXA-48 like producers. Dortet *et al.* have described a sensitivity of 91.3% for OXA-48 like producers, and in another study conducted in France in 2012 the sensitivity of CarbaNP test for OXA-48 like carbapenemase producers was found to be 100% (12,148). In our study out of the five isolates which were negative by the CarbaNP test one was positive for $bla_{\text{OXA-48 like}}$ gene and three were positive for bla_{NDM} gene. The reason for OXA-48 like enzymes showing a positive result in our study could be the use of modified protocol that has demonstrated better sensitivity for OXA-48 like enzymes. It could also be attributed to the predominant presence of the OXA-181 variant of the enzyme which has a higher hydrolytic activity towards carbapenems when compared to the other enzyme variants in the group(38).The reasons for negative result for isolates with bla_{NDM} gene have been the manufacturing of the Mueller-Hinton agar used for sub-culturing the isolates. Dortet *et al.* recommend Mueller-Hinton agar plates by Becton Dickinson, as the optimalmedium which has optimal concentration of zinc for expression of metallo-β--lactamases (MBL)(148). In our study the medium used was the Mueller- Hinton agar by Becton Dickinson, but there were three NDM producers which tested negative with CarbaNP. One isolate which was negative for the CarbaNP test was also negative for PCR, this could be due to the presence of other mechanisms of resistance such as efflux pumps or loss of porin channels. In *E.coli* and *Klebsiella spp.* The mechanisms usually observed are plasmid encoded AmpC enzymes in combination with loss of porin channels OmpK35/36, OmpF or OmpC for *E. coli*(26).

On molecular characterization of these organisms using the multiplex PCR, the predominant gene found was the *bla* $_{NDM}$ gene 40% , which was followed by *bla*$_{OXA48\ like}$ gene in 39% of the isolates. Coexistence of *bla*$_{NDM}$ and*bla*$_{OXA48\ like}$genes was observed in 12% of the isolates. The high prevalence of NDM was comparable to the other studies conducted in the country. As there is a lack of national surveillance data, the prevalence rates can only be compared with the studies published from various centres. SENTRY study conducted in 2006-2007 in various cities in the country found the emergence of NDM and OXA-181 enzymes, prevalence being 51% and 39% respectively(113).SMART study which conducted a worldwide surveillance including the Indian subcontinent revealed that majority of the NDM isolates received for characterization were from the Indian subcontinent(10). Studies conducted at different centres in India also reveal prevalence of NDM from 31% to as high as 90-100% (117,118,120–122)

In our study OXA 48 like enzymes were found common after NDM. Studies conducted in other countries like France and Belgium also reveal OXA-48 and its subtypes as the main carbapenemase type (12,66) Data about OXA enzymes from India is scarce. A study done by Shanthi *et al.* on carbapenem resistant isolates from different clinical samples showed a prevalence of only 1.8% (125). In contrast to this another study by Khajuria *et al.*from central India on carbapenem resistant *Enterobacteriaceae* from urinary isolates showed co-existence of NDM and OXA in 55% of the isolates(9).

KPC production was not observed in any of our study isolates.Nordmann *et al.* in their review stated that though KPC enzymes have been reported from India they are

mainly sporadic outbreaks (101). This is in contrast to the study published by Shanmugam *et al.* where they found the prevalence of 67.4%(123). This variation could be due to sites from where the samples were collected and the smaller sample size in the study. The presence of other genes were also not looked for in this study.

IMP and VIM genes were also not found in any of the isolates in our study. These enzymes have been reported as a common cause of carbapenem resistance by Azim *et al*. in the pre NDM era (149). The prevalence of these enzymes has decreased as the prevalence of NDM has alarmingly increased in the country.

Ten isolates did not show the presence of any one of the five genes. This could be due to the presence of other carbapenemase genes or non-carbapenemse mediated mechanisms(26).

8. Limitations of the study

The exact prevalence of carbapenem resistant *Enterobacteriaceae* in the hospital setting was not estimated since the study included only *K. pneumoniae* and*E. coli* isolates. The prevalence of carbapenem resistant *K. pneumoniae* was also not calculated because, the study included only the isolates from blood stream infections whereas samples from other sources were not included. Thus the burden of carbapenem resistance in invasive infection could only be estimated.

The identification of other mechanisms or resistance in the PCR negative isolates was not performed which could have given insights on rare or novel carbapenemases and non carbapenemase mediated mechanism of resistance in these bacteria.

9. Conclusion

During the period of study, 122 isolates of carbapenem resistant *K. pneumoniae* and *E. coli* were collected from samples received from blood stream infections. Majority of the samples were obtained from hospitalized patients (95%), while few isolates (5%) were received from casualty which might represent community acquired infections also, suggesting the presence of these strains in the community.

Results of the phenotypic rapid test CarbaNP test advocated that production of carbapenemases contributed to the majority of the resistance to carbapenem antibiotics (96%). Among the carbapenemases, New Delhi metallo-β-lactamase was the most prevalent (40%). This was closely followed by Oxaicillinase OXA-48 like enzyme. There was also a coexistence of NDM and OXA-48 like enzymes in 12% of the isolates. Enzymes Imipenemase (IMP), Verona integron metallo β- lactamase(VIM) and Klebsiella pneumoniae carbapenemase(KPC) were looked for but not found in any of the isolates. The OXA-48 like variant in the sequenced isolates was found to be OXA-181 and the NDM variant was NDM-1.

The study isolates were resistant to most of the first line antimicrobial agents. Susceptibility to gentamicin, amikacin, netilmicinand ciprofloxacin was noted in a minority of the isolates. 99% of the isolates were susceptible to colistin which showed good in vitro activity but needs to be use with precaution to prevent emergence of resistance.

The rapid emergence and widespread dissemination of the NDM-1 producing *Enterobacteriaceae*is now well known. The high prevalence of OXA-181 enzyme in

this study could be an indication of the changing epidemiology of the carbapenemases. There is also evidence of co-existence of these genes in the organisms. The alarmingly high prevalence of these enzymes poses a definitive threat for antimicrobial chemotherapy. There is an urgent need for a rigorous antimicrobial policy to prevent emergence of carbapenem resistant strains. Formulating a robust hospital infection control policy and implementing it to prevent the spread of CRE is the most effective way to control the storm caused by these multidrug resistant organisms.

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Standard operating procedure

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1. Introduction

It is necessary to perform the antimicrobial susceptibility test as a routine procedure to predict treatment outcome with these agents.

Implications of susceptibility testing results are as follows:

1. "**Susceptible**": High probability of response to treatment with appropriate dosage and regimen of the antimicrobial agent
2. "**Resistan**t": Treatment with the agent will likely fail or produce no response
3. "**Intermediate**": This may have several meaning
    a. With agent which can be administered at higher dosage, higher dosage can be used for response (e.g., β-lactams)
    b. The agent will be effective in infection localised to area where it is concentrated in the body (e.g. ciprofloxacin in urine)
    c. For body compartment infection where drug penetration is expected to be poor even with inflammation, extreme caution is mandate for the use of the agent (e.g. meningitis)
    d. A buffer zone that prevents strains with borderline susceptibility to be incorrectly categorised as resistant
4. "**Non-susceptible**": Where resistance is rarely encountered and interpretation is available only for the susceptible category

The WHO global report on surveillance for antimicrobial resistance identified lack of standards for methodology as one of the key factors for significant gap in surveillance. The different sections in this book will describe the various methods used for antimicrobial susceptibility testing. For testing susceptibility, the antimicrobial agent contained in a reservoir is allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. A variety of antimicrobial containing reservoirs are developed but the antimicrobial impregnated absorbent paper disk is by far the commonest type used. The disk diffusion method of AST is the most practical method and is still the best method of choice.

All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth. Even automated techniques are variations of the above methods.

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include:

1. Disk diffusion – Stoke's method, Kirby-Bauer method
2. Dilution method – Broth dilution, agar dilution
3. Diffusion and dilution – E-test

2. Requirements for antimicrobial susceptibility testing

In order to derive a meaningful report, appropriate media and quality control strains are prerequisites for antimicrobial susceptibility testing. Interpretation of susceptibility testing is based on those prescribed by various governing bodies using set standards or guidelines. It is important to customise these cut-offs based on the prevailing strains in the particular region and the response to therapy at the prescribed dosage regimen. Among the various interpretative criteria available, those developed by the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) are commonly used by most laboratories. These guidelines are updated every year and it is recommended to follow only the latest guideline based on new evidences.

Media used for susceptibility testing:

1. should support the growth of the organism
2. have low thymidine/thymine content which can interfere with testing of drugs acting on the folic acid pathway
3. should have controlled divalent cation levels (Mg^{2+}, Ca^{2+}, Zn^{2+}) which can interfere with results of aminoglycosides and tetracycline

Mueller-Hinton agar (MHA) at pH of 7.2 to 7.4 is recommended for susceptibility testing for most non-fastidious organisms as it satisfies all of the above criteria and has less batch to batch variations. MHA has also been used and evaluated widely. The media should be poured to obtain thickness of 4 mm. Too thick media will give erroneous resistant and too thin media will give erroneous susceptible reading.

Other media recommended for fastidious organisms are:

1. MHA with 5% sheep blood for *Streptococcus sp*.

2. Haemophilus test medium (HTM) for *Haemophilus sp*.
3. GC agar with 1% supplements for *Neisseria sp*.

The appropriate quality control strains (Table 2.1) should be tested in a similar manner to the test isolates and should give a result within the expected range for the antimicrobials tested weather the zone of inhibition by disk diffusion or MIC. Any result out of range indicates an out of control test and appropriate steps should be taken to rectify any cause before repeating the test.

Table 2.1: Quality control strains recommendation by CLSI:

Organism	**QC strain**
Enterobacteriaceae	*Escherichia coli* ATCC 25922 *Escherichia coli* ATCC 35218 for beta-lactam/beta-lactamase combination *Pseudomonas aeruginosa* ATCC 27853 for carbapenems
Pseudomonas aeruginosa	*Pseudomonas aeruginosa* ATCC 27853 *Escherichia coli* ATCC 35218 for beta-lactam/beta-lactamase combination
Acinetobacter sp.	*Pseudomonas aeruginosa* ATCC 27853 *Escherichia coli* ATCC 35218 for beta-lactam/beta-lactamase combination *Escherichia coli* ATCC 25922 for tetracycline and trimethoprime-sulfamethoxazole
Burkholderia cepacia, Stenotrophomonas maltophilia	*Pseudomonas aeruginosa* ATCC 27853 *Escherichia coli* ATCC 35218 for beta-lactam/beta-lactamase combination *Escherichia coli* ATCC 25922 for chloramphenicol, minocyclin and trimethoprime-sulfamethoxazole
Other non-*Enterobacteriaceae*	*Pseudomonas aeruginosa* ATCC 27853 *Escherichia coli* ATCC 35218 for beta-lactam/beta-lactamase combination *Escherichia coli* ATCC 25922 for chloramphenicol, tetracyclins, sulfonamides and trimethoprime-sulfamethoxazole
Staphylococcus sp.	*Staphylococcus aureus* ATCC 25923 for disk diffusion *Staphylococcus aureus* ATCC 29213 for MIC
Enterococcus sp.	*Staphylococcus aureus* ATCC 25923 for disk diffusion Enterococcus faecalis ATCC 29212 for dilution methods
Haemophilius influenzae *H. parainfluenzae*	*Haemophilus influenzae* ATCC 49247 *Haemophilus influenzae* ATCC 49766 *Escherichia coli* ATCC 35218 for testing amoxicillin-clavulanate
Neisseria gonorrhoeae	*Neisseria gonorrhoeae* ATCC49226

Neisseria meningitidis	*Streptococcus pneumoniae* ATCC 49619 *Escherichia coli* ATCC 25922 for ciprofloxacin, nalidixic acid, minocyclin and sulfisoxazole
Streptococcus pneumoniae and *Streptococcus sp.* (β-haemolyticand Viridans group)	*Streptococcus pneumoniae* ATCC 49619
Anaerobes	*Bacteroides fragilis* ATCC 25285 *Bacteroides thetaiotaomicron* ATCC 29741 For Gram positives: *Clostridium difficile* ATCC 700057 or *Eubacterium lentum* ATCC 43055

3. Disk diffusion by Kirby-Bauer method

This method is recommended by the CLSI for routine testing. The accuracy and reproducibility of this test is dependent on maintaining a standard set of procedures as described here.

3.1 Requirements:

1. Sterile broth medium in 1.5 ml quantities (nutrient broth / Mueller Hinton broth)
2. MHA for Non-fastidious organisms.
3. MHBA for *S. pneumoniae* and other Streptococci
4. HTM for *Haemophilus spp*.
5. GC agar with 1% growth supplements for *Neisseria spp*.
6. Calibrated loop of 2 mm diameter
7. Antibiotic solution
8. Sterile filter paper disks / Commercial disks
9. Pasteur pipettes sterile
10. Cotton swabs sterile
11. Normal saline and / Nutrient broth
12. McFarland BaSO4 turbidity standard 0.5
13. Sterile forceps / needle / disk dispenser
14. 12 x 100 mm sterile test tubes
15. Measuring scales / sliding calipers

16. Table lamp
17. Zone diameter interpretation charts
18. Quality control reference strains (see page no. 4)
19. Discard jar with disinfectant

3.2 Antimicrobials:

Antimicrobials for testing may be available as commercial disk of standard size and strength, or may be prepared in-house from pure substance.

3.2.1 Commercial disk:

1. Commercial disk cartridges should be stored at proper temperature according to recommendations for the particular agent. Labile agents like imipenem, cefaclor, and clavulanic acid combinations should be frozen till day of testing.
2. Remove the sealed packages or cartridges from freezer one or two hours before test to bring it at room temperature. This will prevent condensation forming on the disk.
3. Check for date of expiry before use and discard all expired disk.

3.2.2 Preparation of antimicrobial solution in-house:

Preparation of stock solution:

1. Pure substance of antimicrobial agents may be received as powders or tablets. **Do not use preparations intended for parentral injections.**
2. Powders must be accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile glassware.
3. Standard strains of stock cultures should be used to evaluate the antibiotic stock solution. If satisfactory, the stock can be aliquoted in 5 ml volumes and frozen at -20ºC or -60ºC.
4. Prepare antibiotic solution using the following formula:

$$\textit{Weight}\ (\text{mg}) = \frac{\textit{Volume}\ (\text{mL}) \bullet \textit{Concentration}\ (\mu\text{g/mL})}{\textit{Potency}\ (\mu\text{g/mg})}$$

or

$$\textit{Volume}\ (\text{mL}) = \frac{\textit{Weight}\ (\text{mg}) \bullet \textit{Potency}\ (\mu\text{g/mg})}{\textit{Concentration}\ (\mu\text{g/mL})}$$

3.3 Preparation of inoculum:

The inoculum can be prepared by either the growth method or direct suspension method.

Growth method is usually preferred for non-fastidious organisms and when smooth suspension of the organism cannot be made.

1. With a sterile needle / loop, touch eight or ten well isolated colonies of the same morphological type.
2. Inoculate into 1.5 ml of a sterile suitable broth.
3. Incubate at 35 – 37^{o}C for 2 – 6 hours to produce a bacterial suspension of moderate turbidity.
4. Adjust the turbidity of the broth to McFarland barium sulphate standard 0.5 with sterile saline / broth. This results in a suspension containing approximately 1 to 2 x 10^{8} CFU/ml for *E.coli* ATCC 25922. It should be matched visually in adequate light using a card with a white background and contrasting black lines. Else, a photometric device can be used.

As a convenient alternative to the growth method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies and is recommended for testing fastidious organisms (*Haemophilus* spp., *N. gonorrhoeae, N. meningitidis,* and streptococci), and for testing staphylococci for potential methicillin or oxacillin resistance.

1. Isolated colonies on 18 to 24 hour agar plate from a non-selective medium, such as blood agar are suspended in sterile broth or saline.
2. Adjust to match turbidity of the suspension to 0.5 McFarland standard.

3.4 Inoculation of test plates:

1. Mark the plates into five sections (100 mm petridish) according to the number of antibiotics to be used.
2. Inoculate the plates within 15 minutes of preparation of suspension so that the density does not change.
3. Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level.
4. Inoculate the dried surface of a Mueller-Hinton agar plate by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60^{o} each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

5. The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

3.5 Application of antimicrobial disk:

1. Dispense the predetermined battery of antimicrobial disks onto the surface of the inoculated agar plate within 15 minutes of inoculation of culture.
2. Each disk must be pressed down to ensure complete contact with the agar surface.
3. Make sure that they are no closer than 24 mm from centre to centre. Ordinarily, no more than 5 disks should be placed on a 100 mm plate.
4. Because some of the drug diffuses almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.
5. For in-house prepared antimicrobial solution, a 2 mm calibrated loop is used to deliver 5µl of the solution into 6 mm disk prepared from Whatmann No.2 filter paper, and placed on the surface of the plate.
6. Incubate the plates in an inverted position in an incubator set to 35±2ºC within 15 minutes after the disks are applied.
7. Incubation should be done in ambient air except for *Haemophilus sp., N. gonorrhoea, N. meningitidis* and *Streptococcus sp.*because the interpretive standards were developed by using ambient air incubation, and CO2 significantly alters the size of the inhibitory zones of some antibiotics.

3.6 Reading and interpretation of results:

1. All reading for the test isolate must be taken only when the zone size for the QC organism is satisfactory i.e., within the expected zone size range.
2. Each plates are examined after 16 – 18 hours of incubation. If the test organism is a *Staphylococcus* or *Enterococcus spp.,* 24 hours of incubation are required for vancomycin and oxacillin.
3. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated.

4. The point of abrupt diminution of growth, which in most cases corresponds with the point of complete inhibition of growth, is taken as the zone edge.
5. Measure the diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk.
6. Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate, with reflected light. Zones can be measured easily by holding the petri plate a few inches above a black, non-reflecting background, illuminated with reflected light.
7. If blood was added to the agar base (as with Streptococci), the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed.
8. Transmitted light (plate held up to light) is used to examine the oxacillin and vancomycin zones for light growth of methicillin or vancomycin- resistant *Staphylococcus* and *Enterococcus* colonies, respectively, within apparent zones of inhibition. Any discernable growth within zone of inhibition is indicative of methicillin or vancomycin resistance.

The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified, and retested.

Strains of *Proteus spp.* may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus spp*, the thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored. When using blood-supplemented medium for testing Streptococci, the zone of inhibition of growth should be measured and not the zone of inhibition of haemolysis. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter.

Refer Tables 2A through 2J (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) of the CLSI M100-S24: Performance Standards for Antimicrobial Susceptibility Testing: Twenty fourth Informational Supplement, for interpreting the sizes of the zones of inhibition. Report the organisms as susceptible,

intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.

If disk diffusion tests are performed with fastidious organisms, the medium, QC procedures, and interpretive criteria must be modified to fit each organism. However the general rule of testing remains the same.

Table 3.1: Troubleshooting out-of-range QC for disk diffusion:

Observation	**Probable cause**	**Comments/action**
Many zones too large	Too light inoculum Media depth too thin MHA nutritionally unacceptable	Repeat using McFarland 0.5 turbidity standard Use agar with depth approximately 4 mm Recheck alternate lots of MHA
Many zones too small	Too heavy inoculum Media depth too thick MHA nutritionally unacceptable	Repeat using McFarland 0.5 turbidity standard Use agar with depth approximately 4 mm Recheck alternate lots of MHA
One or more zones too small or too large	Measurement error Transcription error Random defective disk Disk not pressed firmly against agar	Recheck readings Retest. If retest results out of range and no errors detected, initiate corrective action.
One QC strain is out of range, but other QC organisms are in range with the same organism	One QC organism may be better indicator of a QC problem	Retest the strain to confirm reproducibility of acceptable results. Evaluate with alternative strains with known MICs. Initiate corrective action with problem QC strain/ antimicrobial agents.
Two QC strains are out of range with the same antimicrobial agent	Indicates problem with the disk	Use alternative lot. Check storage conditions and package integrity.
Overlapping zones	Too many disk per plate	Place no more than 5 disk per plate for 100 mm petridish and no

		more than 12 disk per plate for 150 mm petridish.
Zone too small for aminoglycosides, clindamycin, macrolides, quinolones Zone too large for penicillins and tetracyclins	pH of media too low	Acceptable pH range is 7.2 – 7.4 Avoid incubation in CO_2
Zone too small for penicillins and tetracyclins Zone too large for aminoglycosides, clindamycin, macrolides, quinolones	pH of media too high	Acceptable pH range is 7.2 – 7.4
Zone for aminoglycosides and tetracyclins too small	Ca^{2+} and/or Mg^{2+} content too high	Use alternative lot of media
Zone for aminoglycosides and tetracyclins too large	Ca^{2+} and/or Mg^{2+} content too low	Use alternative lot of media
Zone ≤ 20 mm of *E. faecalis* ATCC 29212 for sulfonamides	High thymidine content	Use alternative lot of media
E. coli ATCC 35218 zone too small for Amoxicillin-clavulanate or Ticarcillin-clavulanate	Clavulanate is labile. Disk potency lost.	Use alternative lot. Check storage conditions and package integrity.
Zone initially acceptable but slowly decreasing and out of range for beta-lactams	Loss of potency	Use alternative lot. Check storage conditions and package integrity.
Aztreonam, cefotaxime, cefpodoxime, ceftazidime and ceftriaxone zone too large for *K. pneumoniae* ATCC 700603	Spontaneous loss of plasmid encoding the beta-lactamase	New culture must be used
Ampicillin zone too large for *E.coli* ATCC 35218	Spontaneous loss of plasmid encoding the beta-lactamase	New culture must be used

CR no.	Blood no	Name	Hospital nc	Isola	Unit/ward	Age	Sex	cpd	g	ak	netil	cipro	p/t	magne	pb300	imi	mero	cefox	cpi	gene	carbaNP
1	17562	Balli suresh	401410f	2	haem/abm	25	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
2	20068	Lakshmi	600422f	2	haem/aicu	41	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
3	20307	taw marya	161544f	2	uro1/o3w	34	1	1	1	0	1	1	1	1	0	1	1	1	1	1	2 p
4	20346	Magesh	541769d	2	m1/mhdu	38	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
5	20714	Manu mandal	615324f	2	uro/o3w	29	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
6	21080	Sabina khatoon	490699f	2	hpb/sicu	33	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
7	21144	Nazone rahama	623889f	2	casu/op	21	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
8	19984	Siksha kumari	618616f	2	ch2/q5s	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
9	18177	Anita devi	483097f	2	m3/micu	31	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
10	1239	Meghali talukd	414061f	2	haem/l	34	1	1	0	0	0	1	1	1	0	1	1	1	1	1	2 p
11	24275	Naresh gope	631639f	1	uri2/o3w	55	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
12	23617	Rajeshwari	389113f	2	haem/l	22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2 p
13	23898	Alexander	469605f	2	haem/a8	19	2	1	1	1	1	1	1	1	0	1	1	1	1	0	0 p
14	4099	Arup dutta	401465f	1	Haem/a8	44	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
15	22235	Sridevi	604989	2	gec/w	58	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
16	22160	Kalana parveen	629411f	2	haem/a8	8	1	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
17	22609	syed habibu nis	618559f	2	rhc/o5w	18	1	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
18	2613	Rajasekhar	374509f	2	haem/aicu	27	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
19	2449	Shaistavati	389327f	2	cv3/q5s	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	2 p
20	454	Venkatesh	638545d	1	m4/mhdu	53	2	1	1	1	1	1	1	1	0	1	1	1	1	1	0 p
21	2621	Renugopal	913094d	1	haem/w	76	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
22	2984	Bina devi	260815f	1	monc/l	48	1	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
23	2415	ragavan	384180f	2	s2/sicu	32	2	1	1	1	1	1	1	1	0	1	1	1	1	1	0 p
24	16129	Ravi govind raju	448575f	2	haem/aicu	39	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
25	15920	Rajesh kumar	961104c	2	m2/c	37	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
26	708	Dilip Kumar	366837F	2	hpb/hdu	48	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
27	2710	Pithamuthu	300033F	2	haem/A7	55	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
28	3793	Rajendran	394218F	2	M3/mhdu	45	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
29	8072	James jeyachan	626792b	2	haem/micu	72	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
30	17108	Prashanth kum	600984f	2	pmr/q3	22	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
31	12681	Sumathi	633312c	2	neph2/sicu	40	1	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
32	7978	Swati choudhar	422587f	2	Hepa/w	29	1	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
33	12176	Meghali talukd	414061f	2	haem/l	31	1	1	0	0	0	1	1	1	0	1	1	1	1	1	2 p
34	11652	Seelam siva red	399784f	2	hepa/w	21	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
35	12509	venkatamuni	458650f	1	M3/op	83	2	1	0	0	0	1	1	1	0	1	1	1	1	1	1 p
36	13670	Jambulingam	482509d	1	uro1/o3w	74	2	1	0	0	0	1	1	1	0	1	1	1	1	1	0 p
37	24018	Mariamma chac	302236b	2	aem/a8	66	1	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
38	14898	Farzana	850959d	2	nep/h	29	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1 p
39	776	Somit kumar	630912f	2	m3/mhdu	32	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
40	14962	Shanmugammu	474536f	2	m1/micu	65	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1,2 p
41	4286	Anand mohand	398889f	2	haem/A8	17	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
42	4729	Syed Khader	406477f	2	casu/op	84	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
43	29227	debasis	675995f	2	gec/micu	32	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
44	25075	Venkateshwarl	703699d	1	Haem/a8	50	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1 p
45	bv410	Dhiren sarkar	646922f	1	gec/w	53	2	1	1	1	1	1	1	1	0	1	1	1	1	1	2 p
46	29682	Ramesh prasad	670614f	2	ts3/knicu	67	2	1	0	0	1	1	1	1	0	1	1	1	1	1	1,2 p

47	25077	godwin	414404d	2 resp/micu	66	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
48	5342	Pushpdevi baby	415024f	2 ch3/picu	0	2	1	1	1	1	1	1	1	0	1	1	1	1	0 p
49	bm146	Manikrishnan	384351f	2 Med3/e w:	43	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
50	5176	Rajan	960872c	2 heam/A7	61	2	1	1	1	1	1	1	1	0	1	1	1	1	1 N
51	29476	Ram bachan	629549f	1 nep2/h	74	2	1	1	0	0	1	1	1	0	1	1	1	1	1 N
52	30148	Aayan khan	688586f	1 haem/l	10	2	1	1	1	1	1	1	1	0	1	1	1	1	1 N
53	5335	Kallum Durjamr	406163f	2 m3/micu	70	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
54	27937	Shipra modak	664888f	2 m2/mhdu	28	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
55	30490	vijayashanthi	688658f	2 m2/mhdu	22	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
56	5521	Mujeeb	384205f	2 haem/aicu	20	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
57	25513	lila devi	641581f	1 hpb/p2	55	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
58	27922	Rajesh kuppam	671605f	2	17	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
59	31265	Mithlesh gupta	696511f	2 m1/iso	66	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
60	25075	Venkateshwark	703699d	2 Haem/a8	50	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
61	5603	Habida	401124f	2 og3/sicu	38	1	1	1	0	0	1	1	1	0	1	1	1	1	1 p
62	30345	Murali.v	688417f	2 or3/sicu	49	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
63	5734	Lakhan routh	348678f	2 haem/aicu	24	2	1	1	1	1	1	1	1	0	1	1	1	1	1,2 p
64	32057	Roselin	011924c	2 haem/a8	38	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
65	31450	Nagarajan	337325f	2 haem/a4	44	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
66	32537	Sabitri sarkar	704086f	2 hepa/micu	59	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
67	32655	Selvaraja a	293894f	2 s2/p1	40	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
68	30857	Pravathy	464859f	2 hemat/aicu	20	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
69	26257	Surath sonthan	657924f	2 or2/sicu	24	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
70	27079	Mayarani das	638434f	1 m1/iso	40	1	1	1	1	1	1	1	1	0	1	1	1	1	1,2 p
71	29010	Anindita jana	639659f	2 haem/l	26	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
72	31804	Nandaluri	651784f	1 pdon/q5	12	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
73	26796	Preetam yadav	532355d	2 pdon/q5s	5	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
74	bm929	Shanthamani	664965f	2 m3/mts4	65	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
75	32967	Arshiya's baby	695084f	2 N3/l3	0	1	1	1	1	1	1	1	1	0	1	1	1	1	2 N
76	38086	Narayan jhota	426849d	2 haem/aicu	52	2	1	1	1	1	1	1	1	0	1	1	1	1	1,2 p
77	37254	Ibrahim	340966f	2	57	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
78	7053	Vijayakumar	256031d	2 s3/p3	33	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
79	8073	Mandeep kaur	252941f	2 haem/abm	24	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
80	8351	Sunil pramanick	566953d	2 gec/w	63	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
81	10496	Peritandana	441572f	1 m4/mhdu	19	2	1	1	1	1	1	1	1	0	1	1	1	0	1 p
82	33691	Munirathnam n	700992f	2 s3/sicu	68	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
83	22974	Sivakumar	820816d	1 s3/sicu	58	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
84	34215	Leelavathy	713370f	2 pcs/qiw	32	1	1	1	1	1	1	0	1	0	1	1	1	1	1 p
85	34052	Sharatha devi	719819f	1 gec/w	42	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
86	39175	Gayathrie	704720f	2 m2/micu	22	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
87	4352	Raksith	393189f	2 haem/aicu	12	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
88	4994	Sri Krishnan	333534f	2 haem/a8	16	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
89	6308	Shriya rai	409134f	2 haem/aicu	15	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
90	8928	Poornima's bab	429741f	2 nb/l2	0	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
91	10289	Sayandeep mitl	415688f	2 ch2/picu	0	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
92	39993	Ilavarasi	740579f	1 Haem/l	27	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
93	4505	Arjun	409214f	1 gec/w	57	2	1	1	0	0	1	1	1	0	1	1	1	1	1 p

94	36344	Payal amin	369723f	1 haem/aicu	29	1	1	1	0	0	0	1	1	0	1	1	1	1	1 p
95	4208	lilian tirkey	394342f	1 m4/mts4	53	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
96	7583	Minukumar	324106f	1 s3/sicu	21	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
97	4309	Lakshmi	401309f	2 S1/sicu	55	1	1	1	1	1	1	1	1	0	1	1	1	1	0 p
98	36771	Prakash karmak	722455f	2 pdon/q5	9	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
99	34594	Inbaraj	688818f	2 haem/micu	45	2	1	1	1	1	1	1	1	0	1	1	1	1	0 p
100	35168	Nithya S	713478f	2 nu3/micu	23	1	1	1	1	1	1	1	1	0	1	1	1	1	2 p
101	32205	yummun's baby	704114f	2 ch	0	2	1	1	1	1	1	1	1	0	1	1	1	1	1,2 p
102	8472	Megali talukdar	414061f	1 haem/l	34	1	1	1	0	0	1	1	1	0	1	1	1	1	1 p
103	36555	Amvamal.S	6899371f	1 m4/c	55	1	1	1	1	1	1	1	1 *		1	1	1	1	1,2 p
104	37305	Kausally keshari	693871f	1 monc/a7	65	1	1	1	0	0	0	1	1 *		1	1	1	1	1 p
105	24770	mushmi begum	340304c	1 gec/w	75	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
106	37110	ram naresj	727236f	1 med3/e	61	1	1	1	0	0	0	1	1	0	1	1	1	1	0 p
107	25911	Desaraj	485813f	1 Haem/a8	25	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
108	7745	Kaviri. M	422481f	1 rhc/o5w	19	1	1	1	1	1	1	1	1	0	1	1	1	1	0 p
109	35735	Devdas	598704	2 m3/e	68	2	1	1	1	1	1	1	1	0	1	1	1	1	1,2 p
110	5652	Saurav	159795f	1 Haem/l	15	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
111	36204	Pabir k chakrab	680691f	2 hpb/p2	57	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
112	36100	Radha	704166f	1 pdon/q5	3	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
113	21061	Magesh	541769d	2 m1/mhdu	38	2	1	0	1	0	1	1	1	0	1	1	0	1	p
114	36727	Chella muthu M	704353f	2 haem/l	50	2	1	1	1	1	1	1	1	0	1	1	1	1	2 p
115	6005	Padma's baby	415303f	1 pcas/op	0	2	1	1	1	1	1	1	1	0	1	1	1	1	0 p
116	27914	Ravindra kumar	671658f	2 s1/sicu	33	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
117	26281	Manjula's baby	900309f	2 nb/l3	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1 p
118	27033	Duggappu	905504f	2 ch3/q5s	0	2	1	0	0	1	1	1	1	0	1	1	1	1	1 p
119	26786	Gokul	019189g	2 ch1/q5s	1	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
120	27682	Bhuvanan	900379f	2 ch2/picu	4mths	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
121	28284	Sajiya's baby	900563f	2 pds1/q6s	1	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p
122	25880	Jeshua	900083f	2 ch2/q5n	0	2	1	1	1	1	1	1	1	0	1	1	1	1	1 p